# Molecular mechanism of silicon-induced development of Casparian bands in the exodermis of

### Oryza sativa

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### Zusammenfassung

Silizium (Si) ist das zweithäufigste Element im Boden. Es wird von Gräsern in Form von Kieselsäure aktiv aufgenommen und hat viele positive Effekte auf Pflanzen. Einer dieser Effekte ist die Förderung der Bildung des Casparischen Streifens in Oryza sativa. Es wird gezeigt, dass die Wirkung von Si auf die Ausbildung des exodermalen Casparischen Streifens auch bei anderen Pflanzenarten zu finden ist. Die Casparischen Streifen werden zwischen den antiklinalen Zellwänden der endodermalen Zellen aller höheren Pflanzen und bei den meisten Pflanzen darüber hinaus in den antiklinalen Zellwänden der exodermalen Zellen gebildet. Für die Endodermis ist bekannt, dass die Bildung des Casparischen Streifens mit der Ablagerung von Lignin beginnt, gefolgt von Suberin-Lamellen. Die chemische Zusammensetzung exodermaler Casparischer Streifen ist in der Literatur gut beschrieben. Hier wird gezeigt, dass Si die gleichzeitige Ablagerung von Lignin und Suberin-Lamellen in der Exodermis induziert. Monomere von Lignin und Suberin, die dem Phenylalanin-Biosyntheseweg und der Synthese von Fettsäurederivaten entstammen, werden durch ABC-Transporter in den Apoplasten freigesetzt und dort zu Polymeren kondensiert. Der molekulare Hintergrund für die Bildung des Si-induzierten exodermalen Casparischen Streifens ist kaum bekannt. Deshalb wurde zunächst die Funktion von zuvor identifizierten Kandidatengenen des Lignin- und Suberinstoffwechsels und für den ABC-Transporter OsABCG25 untersucht, und für Knockout- (KO) und Overexpressions- (OE) Mutanten für OsABCG25 erfolgreich nachgewiesen. Die veränderte Expression in OsABCG25-Mutanten spiegelte sich auch in der Regulation anderer Kandidatengene des Phenylpropanoid-Biosynthesewegs und des Fettsäurestoffwechsels wider. Auf der Grundlage transkriptomischer Veränderungen wird postuliert, dass OsABCG25 Suberin-Monomere transportiert.

Um weitere Gene zu untersuchen, die für die Si-abhängige Bildung des exodermale Casparischen Streifens von Interesse sind, wurde ein "whole genome GeneChip" verwendet. Es war beabsichtigt, die Funktion dieser neuen Kandidaten-Gene bei der Erzeugung von KO-Mutanten mit Hilfe von "paired modified CRISPR"-Nukleasen (Nickasen) in transgenen Reislinien zu untersuchen. Insgesamt wurden vier transgene Reislinien erzeugt, welche die jeweilige spezifische Nickase in ihrem Erbgut tragen. Zusätzlich wurden zehn homozygote *T-DNA*- oder Retrotransposon KO-Insertionslinien aus T1-Saatgut etabliert. Die neu gefundenen Gene wurden Signaltransduktion, Phenol-/Lipid-Stoffwechsel und ABC-Transportern zugeordnet.

Darüber hinaus zeigte der GeneChip-Ansatz eine Si induzierte Hochregulation von Genen, die an der Fe Homöostase von Reis beteiligt sind, der zur Gruppe der Strategie-II-Pflanzen gehört. Diese Gene sind bekannt dafür, dass sie unter Fe-Mangel verstärkt exprimiert werden. Es wird gezeigt, dass die Kieselsäureernährung die Fe-Konzentration im Apoplast der kortikalen Zellen reduzierte, wo Fe<sup>3+</sup> an Desoxymuginsäure (DMA) gebunden und in den Symplast aufgenommen wird. Die experimentellen Ergebnisse zeigten die Funktion des exodermalen Casparischen Streifens als Barriere für den Fe-Flux aus der Nährlösung in den Apoplast der kortikalen Zellen.

Schlagwörter: Casparischer Streifen, Nickase, CRISPR, Radialer Sauerstoffverlust, Apoplast, Kieselsäure, Eisenstoffwechsel

### **Abstract**

Silicon is the second most abundant element in the soil. It is actively taken up by gramineous plants in form of silicic acid and has several beneficial effects on plants. One of these effects is the promotion of Casparian band (CB) formation in rice. It is shown that the effect of Si on exodermal CB formation also can be found in other plant species. The CBs are developed between the anticlinal cell walls of endodermal cells of all higher plants and in anticlinal cell walls of the exodermis in most plants. For the endodermis, it is known that the CB formation starts with the deposition of lignin and is followed by suberin lamellae. The chemical composition of exodermal CBs are well described in the literature. Here it is shown that Siinduced the simultaneous deposition of lignin and suberin lamellae in the exodermis. Monomeres of lignin and suberin originating from the phenylalanine pathway and fatty acid derivates are released into the apoplast by ABC transporters, where they are condensed to polymers. The molecular reasons and genes involved in Si dependent exodermal CBs formation are poorly understood. Therefore, the function of previously identified candidate genes of the lignin and suberin metabolism and for the ABC transporter OsABCG25 were investigated. The function in Si-induced exodermal CB formation was demonstrated by knockout (KO) and overexpression (OE) mutants for OsABCG25. The altered expression in OsABCG25 mutants was also reflected in the regulation of other candidate genes of the phenylpropanoid pathway and fatty acid metabolism. In conclusion, based on transcriptomic changes in the mutant lines, it is suggested that OsABCG25 transports suberin monomers.

To investigate further genes of interest in the Si dependent exodermal CB formation a whole genome GeneChip approach was used. It was intended to investigate the function of these genes in the generation of KO mutants using paired modified CRISPR nucleases (nickases) in full transgenic rice lines. A total of four paired nickase transgenic rice lines were generated, and additionally ten homozygous T-DNA or retrotransposon KO insertion lines were generated by selfing of T1 plants. New candidate genes were identified being associated with signaling, phenol-/lipid metabolism, and ABC transporter.

In addition, the GeneChip approach revealed a Si-induced upregulation of genes involved in the Fe homeostasis of rice, which belongs to the group of strategy II plants. These genes are known to be upregulated under Fe deficiency. It is shown that silicic acid nutrition reduced the Fe concentration in the apoplast of the cortical cells where Fe<sup>3+</sup> is bound to deoxymugineic acid (DMA) and taken up into the symplast. The experimental results demonstrated the function of the exodermal CB as a barrier for the Fe flux from the nutrient solution into the apoplast of the cortical cells.

Keywords: apoplast, Casparian band, CRISPR, paired nickases, GeneChip, gene expression, exodermis, iron homeostasis, radial oxygen loss, phenylpropanoid metabolism, silicic acid

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### **Abbreviations**

4CL 4-Coumarate-CoA ligase
A. tumefaciens Agrobacterium tumefaciens

ABA cis-absicic acid

ABC ATP-binding cassette

AT Acyltransferase

BAB Berberin-Annilin-Blue

BSTFA N,N-bis(trimethylsilyl)-trifluoracetamide

C3H Coumaroyl-CoA-3-hydroxylase C4H Cinnamate-4-hydroxylase CaMV 35s Cauliflower mosaic virus 35s

Cas9 CRISPR-associated 9

Cas9D10A CRISPR-associated 9 with a modification in aminoacid 10

CB Casparian band CC Central cylinder

CCH p-Coumaroyl CoA 3-Hydroxylase CcoAOMT Caffeoyl-CoA O-methyltransferase

cDNA Copy deoxyribonucleic acid

cef Cefotaxim B
ColE1 Colicin E1

COMT Caffeic acid O-methyltransferase

conc. Concentration

CRISPR Clustered regularly interspaced short palindromic repeats

dest Distilled

DGOAT Diacylglycerol O-acyltransferase

DM Dry matter

DNA Deoxyribonucleic acid drt Distance from the root tip

EDDHA Ethylenediamine-N,N'-bis(2-hydroxyphenylacetic acid

EDTA Ethylenediaminetetraacetic acid

eF1-α Elongation factor 1 alpha

EN Endodermis
ESB1 Ectopic suberin 1

EX Exodermis

FAD Fatty acid desaturase FAE Fatty acid elongase

FAOSTAT Food and Agriculture Organization of the United Nations

Fig. Figure

FY Fluorol Yellow 088

GC-FID Gas chromatography-flame ionization detector

GC-MS Gas chromatography-mass spectrometry

GFP Green fluorescent protein

GOI Gene of interests
gRNA Guide ribonucleic acid

HCCL Hydroxycinnamate-CoA-ligase

hyg Hygromycin B

ICP-MS Inductively coupled plasma mass spectrometry

int Intron

IRRI International Rice Research Institute

KCS β-Ketoacyl-Coa synthase

KO Knockout

LA-ICP MS Inductively coupled plasma mass spectrometry

LB Left boarder
LRR Leucin-rich repeat
MA Mugeneic acid

MCS Multiple cloning side

MES 2-(*N*-morpholino)ethanesulfonic acid

MSU Michigan State University, Rice Genome Annotation Project

NA Nicotianamine

NAA 1-Naphthaleneacetic acid NOS-T Nopalionsynthase-terminator

nt Nucleotide

O. sativa (L.) Oryza sativa ssp. Japonica

OE Overexpression
OPR Outer part of the root

P2x35s 2x Cauliflower mosaic virus 35s promotor

P450 Cytochrome P450

PAL Phenylalanine-ammonia-lyase
PAM Protospacer adjacent motif
PBS Phenylalanine-ammonia-lyase
PCR Polymerase chain reaction

PFG Crop Biotech Institute, Department of Plant Systems Biotech, Kyung

Hee University, Korea

POD Peroxidase

PTS Trisodium 3-hydroxy-5,8,10-pyrenetrisulfonate

qPCR Semiquantitative real time PCR qRT-PCR Semiquantitative real time PCR

RB Right boarder **ROL** Radial oxygen loss RT Room temperature s.e. Standard error **SCL** Sclerenchyma SE Standard error Sm Streptomycin Sp Spectomycin

SPAD Soil-Plant Analyses Development

T-DNA Transfer DNA

Taq Thermus aquaticus

tos17 National Institute of Agrobiological Sciences, Japan

trRNA Tracking RNA

U6os4 Rice U6 promotor sequence

Ubi-int Intron of ubiquitin

UV Ultraviolet WT Wildtype

YSL Yellow stripe like

### **Gene Abbreviations**

docs1 defective in outer cell layer specification 1 Os*ABCG25* ABC transporter 25 of O. sativa (L.) Os*ABCG49* ABC transporter 49 of O. sativa (L.) OsDMAS deoxymugineic acid synthase nicotianamine efflux transporter Os*ENA1* OsFer1/2 Ferritin like 1/2 OsHrz1 Haemerythrin motif-containing RING-& Zinc finger protein 1 OsIRO2 *Iron-related transcription factor 2* Iron related transporter 1 OsIRT1 Low silicon 1 OsLsi1 OsLsi2 low silicon 2 OsLsi3 low silicon 3 OsLsi6 low silicon 6 Nicotianamine aminotransferase 1 OsNAAT1 OsNAS1/2 Nicotianamine synthase 1/2 Os*TOM1/3* Transporter of mugineic acid 1/3 Os*Vit1/2* Vacuolar membrane transporter 1/2 Os*YSL15* Yellow stripe like transporter 15 Os*YSL16* Yellow stripe like transporter 16 Os*YSL2* Yellow stripe like transporter 2 OsYSL9 Yellow stripe like transporter 9 UBQ5 *Ubiquitin 5* hptII hygromycin phosphotransferase

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## General Introduction 1 Silicon in soil and plant

### 1.1 Concentration and available forms of Si

In soils, Si is mostly covalent bound in silicates and oxides. The amount of dissolved silicic acid (Si(OH)<sub>4</sub>) in the soil solution is increasing with pH and temperature and ranges between 2.5-20 mg L<sup>-1</sup> (Epstein, 1994). To ensure sufficient Si supply of rice the sodium acetate soluble Si concentration in soil should be above 40 mg Si kg<sup>-1</sup> (Dobermann and Fairhurst, 2000). Intensive rice cropping reduces the Si soil content over time and may result in Si deficiency because of losses due to leaching and removal with straw (Dobermann and Fairhurst, 2000; Ma and Yamaji, 2006). To substitute Si losses from rice fields, blast furnace slags and convector slags containing CaSiO<sub>3</sub> or MgSiO<sub>3</sub> are used. However, Si solubility in these slags is low and they also contain lime increasing the soil pH. Besides, they may also contain heavy metals. Recently, silica gel was used as Si fertilizer in field experiments, which has a higher solubility and does not affect pH (Klotzbücher *et al.*, 2017).

### 1.2 Silicon uptake in plants

Plants taking up Si in form of Si(OH)<sub>4</sub> are classified as Si accumulators, intermediate types or non- accumulators, corresponding to their Si content 10 mg, 5-10 mg or less than 5 mg Si g<sup>-1</sup> shoot dry weight, respectively (Epstein, 1999*b*; Fleck, 2013). Most dicotyledonous plants are non-accumulators (81 % of the species), like *Arabidopsis thaliana* or *Solanum lycopersicum* (Mitani and Ma, 2005), whereas many monocotyledonous plants are Si accumulators, like wheat (Rains *et al.*, 2006), maize (Mitani *et al.* 2009) or rice (Epstein, 2009). Si excluding species can also be intermediate types or non- accumulators (Tamai, 2003). Excluders (non-accumulators) prevent Si transport to the shoot but not from uptake into the root. The root Si concentration is generally low.

Silicic acid absorption in plants follows Michaelis-Menten kinetics (Rains *et al.*, 2006) with a K<sub>m</sub>-value of 2.83 mg Si L<sup>-1</sup> (equivalent to 0.1 mM).

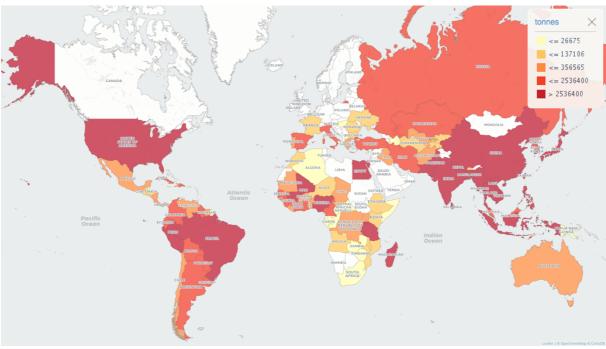
Silicon uptake is mediated by aquaporins classified as nodulin 26-like intrinsic membrane proteins (NIP). Aquaporins facilitate the passive transport of small uncharged solutes (Almagro *et al.*, 2009). There are three aquaporins involved in Si transport from the soil to the seed, which are OsLsi1, OsLsi3, and OsLsi6 (for review see Ma and Yamaji, 2015). Furthermore, the putative anion transporter OsLsi2 is involved in Si uptake. The OsLsi1 is located on the distal side of the exodermis and endodermis, whereas OsLsi2 is found in the proximal side.

### 1.3 Effects of Si in plant growth

Plants benefit greatly from Si application especially if they are able to accumulate Si (Grégoire *et al.*, 2012). Generally, plants have an increased stability and a higher abiotic resistance to stress-factors, like heavy metals, salinity, drought or freezing (Liang *et al.*, 2003; Vinod *et al.*, 2006; Chen *et al.*, 2011; Krishnamurthy *et al.*, 2011). Silicon also mediates a higher resistance to fungal diseases (e.g. powdery mildew, blast), stem borer or grasshopper (Rémus-Borel *et al.*, 2005).

### 1.4 Importance of rice and rice growths conditions

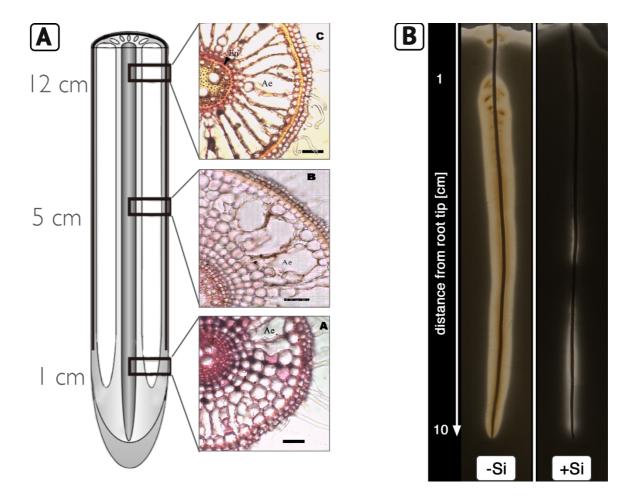
Rice is one of the most produced crops in the world and the nutritional basis for the half of the human population (IRRI, 2002). The production of rice is in a range of 750 million tons per year on 150 million hectares (FAOSTAT, 2017). The main growing areas under paddy soil conditions are in Asia, Africa, North- and South America (Figure 1; Gupta and Toole, 1986). Paddy soil grown rice is the economically most relevant cultivation method in the world. Rice is adapted to flooded field conditions since plant anatomy ensures the oxygen supply to the root.



**Figure 1:** Global production of rice grown on paddy soils. (modified from UN Food and Agriculture Organization, Corporate Statistical Database, 2017).

### 1.5 Rice root anatomy

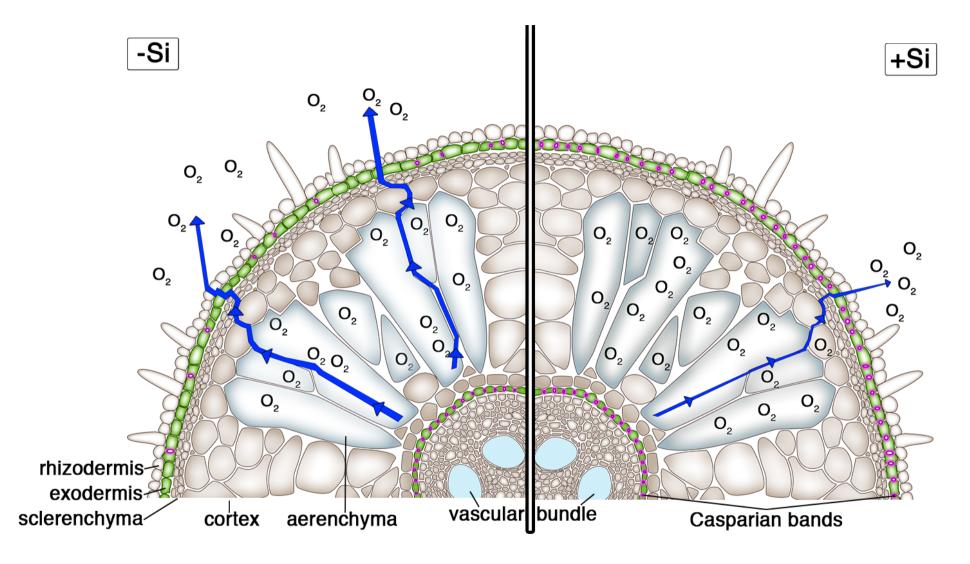
During germination of the rice seeds seminal roots appear growing to a maximal length of 30 cm. After formation of the first nodes adventitious roots emerge, which are thicker and longer than seminal roots and contributing most to nutrient uptake (Yoshida, 1981). Aerobic soil conditions allow a rooting depth up to one meter whereas in anaerobic conditions rooting depth is limited to 40 cm (Yoshida, 1981) because of decreasing oxygen availability. Although roots have an aerenchyma for gas exchange with the atmosphere (Figure 2A). The aerenchyma develops of cortical cells by lysis of cell walls.



**Figure 2:** (A) Formation of aerenchyma in Oryza sativa (L.). Roots are stained with Sudan Fat Red 7B, shown are sections in 1 cm (a), 5 cm (b) and 12 cm (c) distance to the root tip (modified from Nishiuchi et al., 2012). (B) Area of radial oxygen loss (ROL) of rice roots grown with and without Si in the nutrient solution (modified from Fleck et al. 2011).

### 1.6 Radial oxygen loss

The aerenchyma ensures the supply of oxygen to the submerged grown roots (Nishiuchi *et al.*, 2012). Oxygen can diffuse out of the root into the soil solution, called radial oxygen loss (ROL), which is prevented by the formation of the exodermal CB. To visualize the ROL an iron-sulfur agar can be used that turns from black to clear by oxidation of Fe<sup>2+</sup> (Figure 2B). The oxidation power decreases towards the root basis because of the formation of Casparian bands (CB) in the exodermis. Casparian band formation starts about five centimeter behind the root tip and is completed around twelve centimeter behind the root tip. Silicic acid supply enhances the CB formation and thereby reduces the ROL (Figure 2B, 3). Another general effect of CB is the protection of the plant against a nutrient oversupply. Furthermore, it might be speculated that the Si induced increase in Al-tolerance of higher plants is due to this mechanism (Kidd *et al.*, 2001; Singh *et al.*, 2011).



**Figure 3:** Visualization of ROL in rice roots without (-Si) and with (+Si) Si treatment. The missing exodermal CB results in a higher ROL. Silicon treatment enhances the formation of exodermal CB and reduces the ROL thereby.

### 1.7 Casparian band formation in *Oryza sativa* (L.)

Casparian band formation occurs between endodermal root cells of all higher plants (Perumalla and Peterson, 1986) and a majority of plants (e.g. rice) develop an additional CB in between cells of the exodermis (Perumalla *et al.*, 1990). The endodermal CB has several proven functions in nutrient and water uptake and builds an apoplastic barrier mandatory for active nutrient uptake (Enstone *et al.*, 2003). For the endodermal CB it was shown in *Arabidopsis thaliana* that the formation of lignin occurs first and afterwards the deposition of suberin lamellae (Naseer *et al.*, 2012; Doblas *et al.*, 2017). It is not known whether this order also applies for the exodermal CB in rice.

Suberin-lamellae are a deposition of suberin polymers which consist of aromatic and aliphatic compounds. The compounds for suberin formation are provided by the phenylpropanoid pathway and it was suggested that coumaric-, ferulic acid, coumaryl-CoA and feruolyl-CoA are the main aromatic compounds for suberin monomer formation (Franke *et al.*, 2005). The aliphatic compounds of suberin are provided by fatty acid oxidation and are primarily  $\omega$ -hydroxy acids,  $\alpha$ -,  $\omega$ -dicarboxylic acids, fatty acids and alcohols. It was suggested that suberin monomers are excreted by ABC-transporter and that dirigent protein- guided polymerization for the polymer formation is done by oxidation (Davin *et al.*, 1997; Naseer *et al.*, 2012; Hosmani *et al.*, 2013). In contrast to suberin, lignin is a polymer consisting only of aromatic compounds. Sinapic-, ferulic- and coumaric acid are mostly found in lignin while ferulic- and coumaric acid dominate CB (Schreiber, 1996; Boerjan *et al.*, 2003). The formation of endodermal CB occurs early in root growth (1 – 2 cm behind the root tip) while exodermal CB building starts between 6 – 12 cm behind the root tip (Fleck *et al.*, 2011; Nishiuchi *et al.*, 2012).

### 1.8 Si dependent formation of CB in the exodermis of Oryza sativa (L.)

Exodermal CB development is promoted by submerged growth conditions and Si availability in the rhizosphere (Zimmermann *et al.*, 2000; Fleck *et al.*, 2011; Nishiuchi *et al.*, 2012). The presence of Si in the rhizosphere enhances the formation of the exodermal CB. Thus, CB formation starts 2 cm behind the root tip and is finished 6 cm behind the root tip indicating an enhanced formation by Si. This was shown by Berberine-Aniline staining, which dyes the aromatic components of the exodermal CB (Brundrett *et al.*, 1988). However, it is not known whether Si affects also the deposition of suberin lamellae which can be stained by Fluorol Yellow 088.

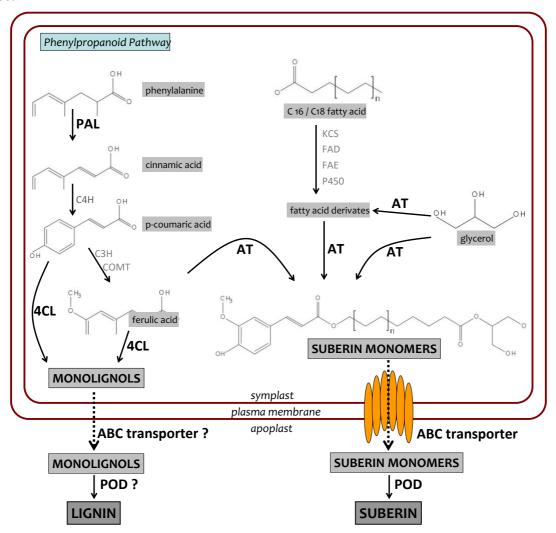
Previous studies had the aim to identify genes involved in the formation of the exodermal CB (Fleck *et al.*, 2011). Thus, a custom-made microarray approach was performed using 256 genes most probably related to the phenylpropanoid-metabolism, fatty acid degradation or suberin / lignin monomer transport. A total of 19 Si-induced genes were identified which were upregulated through Si treatment (Figure 4) and active in the root zone of 4-6 cm behind the root tip where exodermal CB development occurs. However, the function of these genes was not yet proven.

### 1.9 Influence of exodermal CB and Si in nutrient acquisition

The influence of endodermal CB on nutrient uptake is well known (Enstone *et al.*, 2003). Lignin and suberin lamellae build an apoplastic barrier between the endodermal cells. Due to this, it is

possible for the plant to have a higher hydraulic conductivity in the vascular bundle than in the cortex and to establish an enrichment of ions in the vascular bundle to the cortex of roots.

Thus, CB in the endodermis is crucial for active water and nutrient uptake. The endodermal and exodermal CB consist of the same chemical components, suberin and lignin, but functions of the exodermal CB for nutrient fluxes are not known and controversially discussed (Enstone and Peterson, 1997; Sattelmacher, 2001). In experiments using apoplastic tracer such as trisodium 3-hydroxy-5,8,10-pyrene- trisulfonate or *cis*-absicic acid, CB did not reduce flux of tracers into the apoplast (Zimmermann and Steudle, 1998; Freundl *et al.*, 2000). However, it was shown that silicic acid supply in nutrient solution grown *O. sativa* can reduce the uptake of several elements like As, Al, Ca, Fe, Mg, Mn and P (Ma and Takahashi, 1990; Ma *et al.*, 2001; Dufey *et al.*, 2013). The causal relationships for these observations are not known but it is hypothesized that the Si related CB formation in the exodermis is involved. For rice grown in nutrient solution it is suggested that Fe<sup>III+</sup> is predominantly bound to deoxymugineic acid (DMA)in the root apoplast and taken up in this complex by specific transporters (Ogo *et al.*, 2014*a*; Senoura *et al.*, 2017). Furthermore, genes involved in biosynthesis of DMA via nicotianamine and excretion of DMA are known as part of the Fe homeostasis mechanisms in rice.



**Figure 4:** Phenylpropanoid- metabolism for synthesis of the Casparian band (adapted from Fleck, 2013). 4CL= 4-coumarate-CoA ligase, AT= acyltransferase; C3H= coumaroyl-CoA-3-hydroxylase; C4H= cinnamate 4-hydroxylase; COMT= caffeic acid O-methyltransferase; PAL= phenylalanine ammonia-lyase; POD= peroxidase.

### 2 Hypotheses

The current study has the aim to increase the knowledge on the composition of Si induced exodermal CB and on genes involved in this process. In previous studies using a costum made microarray candidate genes were identified but their function was not yet proven. Consequently, the hypotheses of this thesis are as follows:

- 1) Suberin lamellae occur from the very beginning of Si induced CB formation in the exodermis.
- 2) The previously identified candidate genes are involved in the exodermal CB development.
- 3). Comparison of whole genome expression profiles reveals further genes significant for Si induced CB formation and metabolic changes.
- 4) The exodermis has a function in nutrient acquisition.

### 3 Central methods

Since 2004 *O. sativa* is sequenced by the International Rice Genome Sequencing Project and is annotated by the *Rice Genome Annotation Project* with 389 Mb and 55986 predicted gene locations and an overall good annotation (IRGSP, 2005; Kawahara *et al.*, 2013). For transcriptomic studies, whole-genome microarrays are available, like the Affymetrix Genechip, which covers 95 % of the predicted genes. Thus, an Affymetrix GeneChip will be used to identify possible candidate genes involved in the Si induced formation of exodermal CB. The function of candidate genes will be investigated by knockout and overexpression mutants. If not available, KO mutants will be created by the CRISPR technique and *Agrobacterium tumefaciens* induced rice callus transformation.

## Chapter I

4 Silicon Promotes Exodermal Casparian Band Formation in Si-

### Acchumulating and Si-Excluding Species by Forming Phenol

### **Complexes**

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<u>Sascha Schulze:</u> Performed the experiments; analyzed the data; contributed reagents/materials/analysis tools; wrote the manuscript.

Martin Hinrichs: Performed the experiments; analyzed the data; contributed reagents/materials/analysis tools; wrote the manuscript.

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38 <u>Friedrich Waßmann:</u> Analyzed the data; contributed reagents/materials/analysis tools.

40 <u>Lukas Schreiber:</u> Conceived and designed the experiments; contributed reagents/materials/analysis tools.

Manfred K. Schenk: Conceived and designed the experiments; wrote the manuscript.



RESEARCH ARTICLE

## Silicon Promotes Exodermal Casparian Band Formation in Si-Accumulating and Si-Excluding Species by Forming Phenol Complexes

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We studied the effect of Silicon (Si) on Casparian band (CB) development, chemical composition of the exodermal CB and Si deposition across the root in the Si accumulators rice and maize and the Si non-accumulator onion. Plants were cultivated in nutrient solution with and without Si supply. The CB development was determined in stained root cross-sections. The outer part of the roots containing the exodermis was isolated after enzymatic treatment. The exodermal suberin was transesterified with MeOH/BF3 and the chemical composition was measured using gas chromatography-mass spectroscopy (GC-MS) and flame ionization detector (GC-FID). Laser ablation-inductively coupled plasma-mass spectroscopy (LA-ICP-MS) was used to determine the Si deposition across root cross sections. Si promoted CB formation in the roots of Si-accumulator and Si non-accumulator species. The exodermal suberin was decreased in rice and maize due to decreased amounts of aromatic suberin fractions. Si did not affect the concentration of lignin and lignin-like polymers in the outer part of rice, maize and onion roots. The highest Si depositions were found in the tissues containing CB. These data along with literature were used to suggest a mechanism how Si promotes the CB development by forming complexes with phenols.

## CrossMark

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### Introduction

Silicon (Si) as the second most abundant element in the earth crust is nearly everywhere available and is taken up by plants in its soluble form silicic acid [1]. The plant kingdom can be divided into Si accumulators, intermediate type species and Si non-accumulators according to their shoot Si concentration, which ranges from 0.1 up to 10% on a dry weight basis [2]. Si accumulating species are found among the diatoms and the horsetails (*Equisetum*), to which Si



is essential, and among the grasses, including rice, maize and other cereals [3,4]. The high Si concentration in the shoot of rice and maize plants is enabled by transporters that are located in the roots and facilitate the transport of silicic acid towards the xylem [5–7]. Despite the high concentration of Si in the leaves, where it is deposited as silica,  $SiO_2$ , Si is not considered essential for higher plants as defined by Arnon and Stout (1939) [8]. However, Si enhances plant growth and alleviates several biotic and abiotic stresses [9, 10] and therefore Si is designated a beneficial or quasi-essential element [3].

In rice plants, Si was shown to decrease the radial oxygen loss from the root to the anaerobic environment and this was accompanied by a promotion of the Casparian band (CB) formation in the exodermis and endodermis [11]. Moreover, the transcription of genes related to the synthesis of lignin and suberin, the main components of the CB, was increased by Si supply. Suberin is an apoplastic biopolymer composed of the aliphatic compounds  $\omega$ -hydroxy acids,  $\alpha$ ,  $\omega$ -dicarboxylic acids, fatty acids, and alcohols, as well as the aromatic phenylpropanoids p-coumaric and ferulic acid [12]. Lignin is a complex aromatic heteropolymer, which is composed mainly of the lignin monomers p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol [13, 14].

Although the promotive effect of Si on CB formation was clear, the underlying mechanism remained unclear. The fortification of the CB might be related to the availability of Si in the root tissue and if so, the Si effect should not be restricted to plants that accumulate Si in the shoot but also to Si non-accumulators. To clarify this, we investigated the effect of Si supply on the development of CB in rice and maize plants as well as in the Si non-accumulator onion (*Allium cepa*). The impact of Si on the chemical composition of the exodermal suberin was analyzed and quantified by gas chromatography-mass spectroscopy (GC-MS) and flame ionization detector (GC-FID), lignin amounts were determined photometrically. Moreover, the use of laser ablation-inductively coupled plasma-mass spectroscopy (LA-ICP-MS) enabled us to analyze the Si distribution within root cross sections of the three species. Finally, we propose a model explaining how Si could promote CB formation by interacting with chemical compounds of the CB.

### **Materials and Methods**

### Plant material and growth conditions

Rice (Oryza sativa 'Oochikara') seeds were germinated in tap water for 7 d and then placed between two layers of filter paper standing in tap water for additional 7 d. Maize (Zea mays 'Helix') seeds were germinated between two layers of filter paper standing in tap water for 5 d. Onion (Allium cepa 'Hercules I hybrid') bulbs and nug (Guizotia abyssinica) seeds were cultivated in peat substrate for 5 d and 14 d, respectively, and then roots were washed with tap water. Subsequently 5 plants of rice, maize, onion, nug, and Tradescantia virginiana were transferred to 5 L pots with nutrient solution, which was aerated for all plants except for rice plants. The composition of the nutrient solution was in mM: 1.43 NH<sub>4</sub>NO<sub>3</sub>, 0.32 NaH<sub>2</sub>PO<sub>4</sub>, 0.51 K<sub>2</sub>SO<sub>4</sub>, 1 CaCl<sub>2</sub>, 1.6 MgSO<sub>4</sub>; in μM: 1.82 MnSO<sub>4</sub>, 0.03 (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>, 9 H<sub>3</sub>BO<sub>3</sub>, 0.6 ZnSO<sub>4</sub>, 0.15 CuSO<sub>4</sub> and 35.81 Fe as sequestrene (Fe-EDDHA). In the +Si treatment, the Si concentration was adjusted to 1.07 mM (which is equivalent to 30 mg L<sup>-1</sup>) and Si was applied as silica gel (Roth, Karlsruhe, Germany), while the control treatment (-Si) received no Si. The pHvalue was adjusted to 6.0 by addition of  $m H_2SO_4$  and KOH. The plants were cultivated in a growth chamber (photoperiod: 16 h light, 8 h dark; temperature 25°C day / 20°C night; relative humidity 75%; light intensity 220 μmol m<sup>-2</sup> s<sup>-1</sup>) and nutrient solution was changed every 3 d. All plants were harvested after 21 d in nutrient solution and 2 cm root zones were sampled and stored in 70% ethanol at 4°C. Shoot and root were separated and dried at 60°C for 4 d and



ground. The root growth rate was determined 1 d before harvest by marking the roots 2 cm behind the tip with a waterproof marker and measuring the growth after 24 h.

### Silicon analysis

For determination of the Si concentration in the shoot and root, 200 mg dried and ground plant matter was digested in 3 ml 65% HNO<sub>3</sub>, 2 ml H<sub>2</sub>O and 2 ml 30% H<sub>2</sub>O<sub>2</sub> in a microwave for 12 min at 190°C and then diluted with 20 ml 10% NaOH and neutralized with HNO<sub>3</sub> [15]. The Si concentration in the extract and in nutrient solution was determined photometrically at 811 nm after addition of 250  $\mu$ l dye reagent (0.08 M sulphuric acid and 2% ammonium heptamolybdate), 250  $\mu$ l 3.3% tartaric acid and 250  $\mu$ l 0.4% ascorbic acid to 50  $\mu$ l of samples [16].

### Histochemical examination of roots

From each of the 4 replicates, 5 roots without lateral roots were taken for cross sectioning and from 5 cross sections 20 cells each were used for microscopic examination, so the degree of development of CB was calculated on basis of 400 cell walls per treatment. The root zones, where CB initiated under-Si conditions (zone A), and older root zones 4 cm behind (zone B) were harvested. Root zone A was at 0–2 cm distance from the root tip (drt) (rice, onion), 2–4 cm drt (maize), 6–8 cm drt (*Tradescantia*), and at 8–10 cm drt (nug), root zone B was at 4–6 cm drt (rice, onion), 6–8 cm drt (maize), and at 12–14 cm drt (nug). About 0.5 cm from both edges of the 2 cm-zone were removed and free hand cross sections were made from the remaining middle part of the root zone.

For detection of CB, free hand cross sections of plant roots were stained with 0.1% (w/v) berberine hemi-sulphate for 60 min and with 0.5% (w/v) aniline blue for further 30 min [17]. Stained sections were mounted in 0.1% (w/v) FeCl<sub>3</sub> in 50% (v/v) glycerine and examined using an Axioskop fluorescence microscope (Zeiss, Jena, Germany) with UV illumination and excitation filter G 365, chromatic beam splitter FT 395 and barrier filter LP 420. Pictures were taken with the AxioCam MRc (Zeiss) and picture recording software (AxioVision Ac, Version 4.4, Zeiss). Under UV light, suberin exhibited a blue-white colour. The development of CB in the anticlinal exodermal cell walls was determined and allocated to one of four stages: 0% (stage I), 0–25% (II), 25–50% (III) and 50–100% (IV) development of CB in the anticlinal cell wall of the exodermis.

For comparative visualization of CB and suberin lamellae (\$5 Fig), roots of onion, rice and maize were infiltrated with fixation solution containing 3.7% (v/v) formaldehyde, 3.7% (v/v) glutaraldehyde in PBS buffer (137 mM NaCl, 2.7 mM KCL, 10 mM Na<sub>2</sub>HPO<sub>4</sub> 1.8 mM KH<sub>2</sub>PO<sub>4</sub>) for 12 h at 8°C in glass vials. After fixation roots were dehydrated using successive baths of 70, 80, 90 and 100% of ethanol for 1 h each on a radial shaker. Then, dehydrated roots were incubated for 1 h at 39°C in a 1:1 mixture of ethanol and steedman's wax (9:1 Polyethylenglycol: 1-Hexadecanol). For embedding roots were infiltrated with 100% steedman's wax overnight, gently shaking at 39°C. On the next day the wax was changed 2 times with 1 h incubation. Fixed roots were then placed vertically into a holder and chilled at RT overnight. From the imbedded roots, 10 μm serial sections were cut using a microtome (Hyrax M55, Zeiss, Jena, Germany) placed on glass slides (SuperFrost®, Carl Roth, Karlsruhe) on a drop of water to unfold on 32°C (Medox Type 14801 heating plate). Afterwards, water was removed and slides were dried overnight followed by de-waxing on 40°C using 100% pre-warmed ethanol. Staining was done directly on the glass slides. The berberine-aniline blue-staining was applied as described above with the exception of 3 x 5 min each washing steps after berberine hemi-sulphate staining and 3 x 10 min each washing steps after 0.5% aniline blue staining. For fluorol yellow 088 staining a modified protocol from Brundrett, Enstone & Peterson (1988) [17] and



Lux et al. (2005) [18] was applied. A fresh 0.001% solution of fluorol yellow 088 was prepared in lactic acid, then heated to  $70^{\circ}$ C and filtrated using a 20  $\mu$ m filter.

Pictures were taken with the AxioCam MRc (Zeiss) and picture recording software (AxioVision Ac, Version 4.4, Zeiss). Under UV light, suberin exhibited a blue-white colour. The development of CB in the anticlinal exodermal cell walls was determined and allocated to one of four stages: 0% (stage I), 0-25% (II), 25-50% (III) and 50-100% (IV) development of CB in the anticlinal cell wall of the exodermis.

Microscopy of berberine-aniline blue staining of CB was done using a Axioplan I fluorescence microscope (Zeiss, Jena, Germany) and picture recording software (AxioVision Ac, Version 4.8, Zeiss). Fluorol Yellow 088 staining was visualized using a GFP filter with an excitation filter BP 485, chromatic beam splitter FT 510 and barrier filter LP 520. Suberin should appear in a bright yellow / green fluorescence signal. Pictures were adjusted in brightness using the ImageJ-software 1.50a, additionally fluorescence pictures of onion were equally enhanced by 0.1% true colour contrast.

### Cell wall isolation and preparation for suberin and lignin analysis

The root surface of harvested root zones was scanned using WinRHIZO software (Regent Instruments Inc., Quebec, Canada) in order to relate components of the outer part of the root to root surface. The cell wall isolation and preparation was performed as described in detail previously [19]. Briefly, root zone B of rice, maize and onion plants were washed with distilled water and then incubated at room temperature for 4 d in 1 ml enzyme solution (0.1 M citric acid monohydrate, 1% pectinase (v/v), 1% cellulase (v/v), 0,1% NaN<sub>3</sub>), which was renewed daily. After enzymatic digestion the non-degradable outer part of the root comprising the exodermal cell wall fraction was separated from the tissue containing the stele by use of two forceps under a binocular. The exodermal cell wall material was incubated in enzyme solution for another 2 d to remove any residual cortex material. Afterwards the isolates were washed with distilled water and incubated in borate buffer (0.01 M sodium borate, pH 9) for 2 d.

Isolated cell wall material, dried for  $1\ d$  at  $60^{\circ}$ C, was extracted for  $5\ d$  with a  $1:1\ mixture$  of chloroform and methanol, which was changed daily. After the extraction the isolated samples were dried for  $2\ h$  in the desiccator over silica gel. The dry weight was determined directly before suberin and lignin analysis of the isolated samples.

### Suberin analysis

For transesterification, the dried isolates were incubated for 16 h in 1 N methanolic boron trifluoride (MeOH/BF<sub>3</sub>; Fluka) at 70°C. Saturated NaCl was added to stop the transesterification reaction and to advance the following phase separation. Dotriacontan ( $C_{32}$  alkane, 10.025 mg / 50 ml) was added as internal standard to each sample. The soluble hydrophobic components were extracted by adding chloroform. The chloroform phase was transferred to a new vial and extraction was repeated three times. The extract was dried with water free Na<sub>2</sub>SO<sub>4</sub> and the volume was reduced to 50  $\mu$ l by evaporation under N<sub>2</sub> flow.

Samples were derivatized in 20 µl BSTFA (N,N-bis(trimethylsilyl)-trifluoracetamide; Machery-Nagel, Düren, Germany) and 20 µl dry pyridine (GC-grade, Merck, Darmstadt, Germany) for 40 min at 70°C. Pyridine catalyzed the derivatization reaction and BSTFA masked free hydroxyl- and carboxylgroups forming the corresponding trimethylsilyl derivatives [20]. Samples were analyzed by gas chromatography (GC; Type: 6890N, Agilent Technologies, Santa Clara, USA) and mass spectroscopy (MS; Type: 5973N, Agilent Technologies). The GC and MS analysis was performed as described in detail previously [21]. The quantification of the



monomers was performed using a gas chromatograph combined with a flame ionization detector. Four replicates of each plant species were used.

### Lignin analysis

0.5 mg of isolated and extracted cell wall material was solubilized in 1 ml acetyl bromide/acetic acid (1:3, v/v) in a loosely capped glass tube for 30 min at 70°C. Afterwards the tube was cooled down to 15°C and 0.9 ml NaOH and 5 ml glacial acetic acid were added to hydrolyze excess acetyl bromide. Bromine (Br) and polybromide were destroyed by adding 0.1 ml hydroxylamine-HCl. The solution was diluted to a total volume of 10 ml with acetic acid. Within 10–15 min after cooling down the absorption was read at 280 nm with a fused glass microplate (9 mm layer thickness) on a microplate reader ( $\mu$ Quant, BioTek Instrument, Inc., Winooski, USA). The resulting absorbance was multiplied by 1.11 to correct for a cuvette with 10 mm thickness, where a lignin concentration of 14  $\mu$ g ml<sup>-1</sup> led to an absorption of 0.343 [22]. Four replicates of each plant species were used.

### Embedding and sectioning of roots for LA-ICP-MS

Root zone B of rice, maize and onion plants grown in +Si nutrient solution were embedded using the Steedman's wax protocol [23] in a modified form. Roots were fixed in freshly prepared Farmer's fixative (3 parts ethanol + 1 part acetic acid) at 4°C overnight. Roots were then dehydrated under rotation for each 2 h in 75%, 85%, 95% and 100% ethanol, respectively. Molten Steedman's wax (9 parts poly (ethylene glycol) distearate (SigmaAldrich, St. Louis, USA) + 1 part 1-hexadecanol (SigmaAldrich)) was mixed 1:1 with ethanol and roots were incubated in the mixture at 38°C overnight. Roots were then incubated three times at 38°C for 2 h each in pure Steedman's wax. Afterwards, roots were divided in 3 mm pieces and embedded in Steedman's wax in TurbOflowII molds and cassettes (McCormick Scientific, St. Louis, USA). The wax was allowed to solidify overnight at room temperature. The wax blocks were cut with a Hyrax M55 rotary microtome (Zeiss, Jena, Germany) into slices of 20, 50 and 100 µm. Wax slices were dissolved by addition of ethanol and root sections were washed several times by exchanging ethanol until complete removal of the wax.

### Laser ablation-inductively coupled plasma-mass spectroscopy

Root sections floating in ethanol were transferred to Tin (Sn) foils (Elementar Analysensysteme GmbH, Hanau, Germany) placed on microscopy glass slides. Evaporation of the ethanol fixed the root sections to the foil, which allowed the use of a laser for ablation. For rice and maize, 100  $\mu$ m thick root sections were used and for onion, 50  $\mu$ m thick slices were used. Root tissue was ablated with the solid state NYAG-laser UP193 SS (New Wave Research Co. Ltd., Cambridge, England). The laser beam was adjusted to a diameter of 75  $\mu$ m and energy of 2.5 J cm<sup>-2</sup> for rice and maize and to a diameter of 50  $\mu$ m and energy of 4.0 J cm<sup>-2</sup> for onion. The pulse length was 1 s and the frequency was 10 Hz. The ablation chamber was coupled to the ICP-MS torch with a tygone<sup>48</sup> tube and was filled with carrier gas at a flow rate of 0.25 L min<sup>-1</sup>. After the chamber was passed the flow rate was increased with makeup gas to 1.2 L min<sup>-1</sup>. <sup>13</sup>C and <sup>28</sup>Si signals were detected using the quadrupole ICP-MS 7500 CX (Agilent Technologies, Santa Clara, USA). The Si abundance in the root tissue was calculated as <sup>28</sup>Si: <sup>13</sup>C ratio and expressed in relation to the maximum value, which was set to 1.0.



### Statistical analysis

All treatments were replicated four times and mean of the treatments were compared with t-test using R software [24]. For comparison of the developmental stages of CB, a cumulative link mixed model was calculated with p < 0.05 using the package ordinal in R software.

#### **Results**

### Plant growth, Si concentrations and Casparian bands

The shoot dry weight of rice and maize plants was not affected by Si supply, while Si supply increased the shoot dry weight of onion plants (Fig 1A). Similarly, the root dry weight of onion plants was slightly higher in +Si than in-Si plants, while the root dry weight of rice and maize plants did not differ between the Si treatments (Fig 1B). The root growth rate was highest for maize and lowest for onion, however, root growth of no plant species was affected by Si supply (Fig 1C).

The Si concentration in the shoot of rice and maize plants was clearly increased when Si was supplied, while in onion shoots the Si concentration did not differ between the Si treatments (Fig 2A). In contrast, all species accumulated additional Si in the roots when Si was supplied (Fig 2B).

The formation of exodermal CB started in different distances from the root tip (zone A). In rice and onion roots the CB was already developed at 0–2 cm drt, while in maize roots CB initiated at 2–4 cm drt and even later in *Tradescantia* (6–8 cm drt) and nug (8–10 cm drt) (Fig 3; S3 and S4 Figs). Si supply promoted the formation of exodermal CB in all plant species in both, young (zone A) and older root parts (zone B: 4–6 cm drt for rice and onion; 6–8 cm drt for maize; 12–14 cm drt for nug). The development of suberin lamellae was similar to the development of CB in rice, maize and onion exodermis (S5 Fig).

### Suberin and lignin

The composition and quantity of suberin was determined in the outer cell layers comprising the exodermis in the root zone B of rice, maize and onion roots. In rice and maize roots the total suberin was decreased by Si supply. This was due to reduced amounts of the phenolic compounds p-coumaric acid and ferulic acid (Fig 4A and 4B). The sum of the aliphatic suberin monomers was not decreased by Si supply, although single aliphatic compounds were affected by Si supply in rice and maize (S1 Fig). In contrast, the Si treatments did not affect any suberin fraction in onion roots (Fig 4C). Moreover, ferulic acid was the only phenolic suberin compound in the onion root, while p-coumaric acid was not detected.

The content of lignin and lignin-like polymers in the outer cell layers of the root comprising the exodermis and the sclerenchyma in rice, and the exodermis in maize and onion was not affected significantly by Si supply (Fig 5). The lignin concentration in rice was about two times higher than in maize and onion roots.

### Distribution of Si in the root

The Si distribution in root zone B of rice, maize and onion roots was determined by analyzing the  $^{28}$ Si: $^{13}$ C ratio using LA-ICP-MS. The Si abundance was expressed in relation to the maximum value which was set to 1.0. In the rice root, the Si abundance was highest in the outer cell layers comprising the exodermis and the sclerenchyma and gradually decreased towards the central cylinder (Fig 6A). In the maize root, the Si signal was highest in the endodermis, while the Si signal was only slightly higher in the exodermis than in the cortex (Fig 6B). The highest



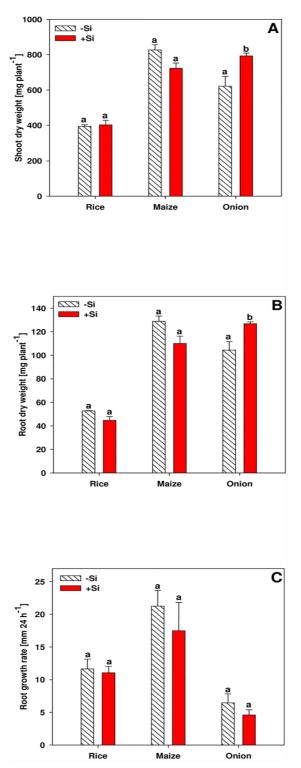


Fig 1. Shoot (A) and root (B) dry weight and root growth rate (C) of rice, maize and onion plants as affected by Si supply. Data are mean  $\pm$  s.e., n = 4. Different letters indicate a significant difference between Si treatments of a species; t-test with p < 0.05.

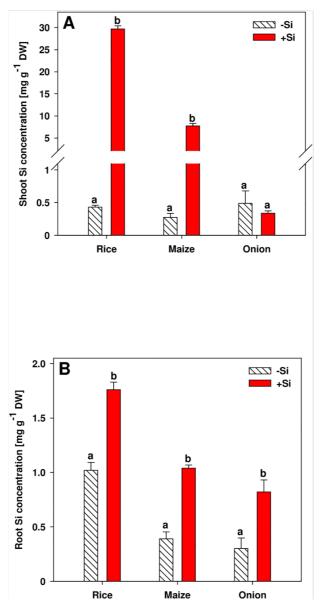


Fig 2. Silicon concentration in shoot (A) and root (B) of rice, maize and onion plants as affected by Si supply. Data are mean  $\pm$  s.e., n = 4. Different letters indicate a significant difference between Si treatments of a species; t-test with p < 0.05.

Si concentration in the onion root was found in the outer region comprising the exodermis, while it was clearly lower in the cortex as well as endodermis and central cylinder (Fig 6C).

### **Discussion**

### Plant growth, Si concentration and Si distribution

Silicon supply did not affect the growth of rice and maize plants, while onion shoot and root dry weight was increased when grown in +Si nutrient solution (Fig 1A and 1B). It is well-known that Si may enhance the growth of rice plants when cultivated in rice paddy fields



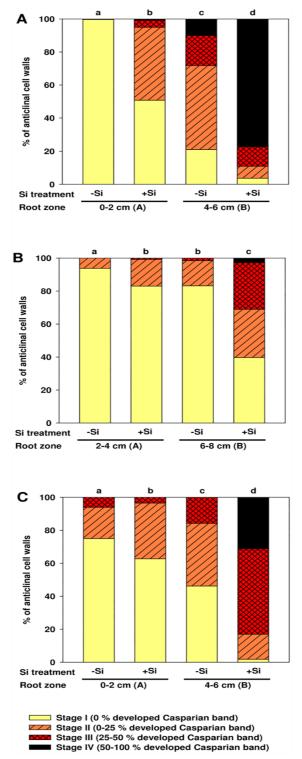


Fig 3. Development of Casparian bands (CB) in the exodermis of rice (A), maize (B) and onion (C) roots as affected by Si supply. Root zone where the formation of CB initiated was defined as root zone A, which was at 0-2 cm drt for rice and onion, and at 2-4 cm drt for maize. Root zone B started 2 cm behind root zone A. Exodermal CB were classified into stages I-IV according to 0, 0-25, 25-50 or > 50% of the length of the anticlinal cell wall with developed CB. n = 4. Different letters indicate significant difference between Si treatments and root sections of a species; cumulative link mixed models with p < 0.05.



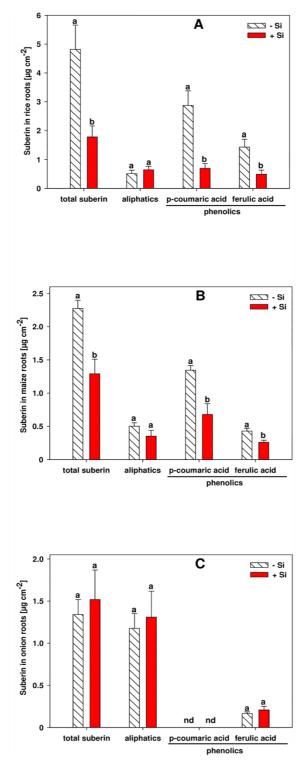


Fig 4. Total, aliphatic and phenolic suberin amounts in the outer cell layers comprising the exodermis of root zone B in rice (A), maize (B) and onion (C) as affected by Si supply. Amounts were determined via GC-FID. Root zone B was at 4-6 cm drt in rice and onion and at 6-8 cm drt in maize roots. Data are mean  $\pm$  s.e., n=4. Different letters indicate a significant difference between Si treatments of a suberin fraction; t-test with p < 0.05. t-nd enot detected.

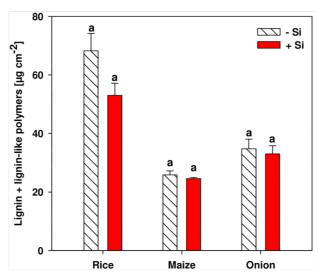


Fig 5. Concentration of lignin and lignin-like polymers in the outer cell layers of the root comprising the exodermis and the sclerenchyma in rice, and the exodermis in maize and onion as affected by Si supply. Amounts were determined photometrically. Root zone B was at 4-6 cm drt in rice and onion and at 6-8 cm drt in maize roots. Data are mean  $\pm$  s.e., n = 4. Different letters indicate a significant difference between Si treatments of a plant species; t-test with p < 0.05.

[3, 25, 26]. However, a growth promotion of rice plants by Si supply was not observed in other studies with rice cultivated in nutrient solution [11]. The reason might be the optimal cultivation conditions in nutrient solution in a climate chamber where no stress could be alleviated by Si application [3]. By contrast, a growth promotion of onion plants is in contradiction to other reports in literature [25]. A higher initial dry matter could be the reason for higher final dry matter yield in the +Si treatment, since the onion bulbs were inhomogeneous. This is further supported by the fact that Si did not affect root growth rates in onion and in the other two plant species (Fig 1C), which is in line with previous data [11].

The Si concentrations in the shoots of rice, maize and onion plants grown in-Si nutrient solution were on a similar level, while shoot Si concentrations were clearly enhanced in rice and maize plants when Si was supplied (Fig 2A). By contrast, onion did not accumulate additional Si in the shoot when grown in +Si nutrient solution. This is in line with literature that classified onion as Si non-accumulator [25]. Moreover, rice has a high ranking in the extensive list of Hodson et al. [4], who ranked 735 plant species according to their shoot Si concentration, followed by maize, while the onion family *Amaryllidaceae* has a very low ranking. The Si concentrations in the root, however, were enhanced by Si supply in all species (Fig 2B).

The concentration of Si in the rice roots was generally highest in the outer part of the root comprising the exodermis and the sclerenchyma (Fig 6A). Silica depositions in the rice root were found in the tangential and in the radial cell walls of the endodermis [27], while Si accumulation in other studies was reported not only in the endodermis but also in the exodermis of rice roots [28, 29]. More recently, Moore et al. [30] confirmed silica deposition in the cell walls of both, endodermis and exodermis, as well as in the sclerenchyma.

The highest Si concentration in the maize root was found in the endodermis, while in onion roots, the exodermis contained the most Si (Fig 6B and 6C). Differently from rice, the Si distribution in maize and onion roots had not been reported before. However, Si accumulation in the endodermis has also been reported for other grasses, including sorghum, barley, oats, and wheat [31, 32].



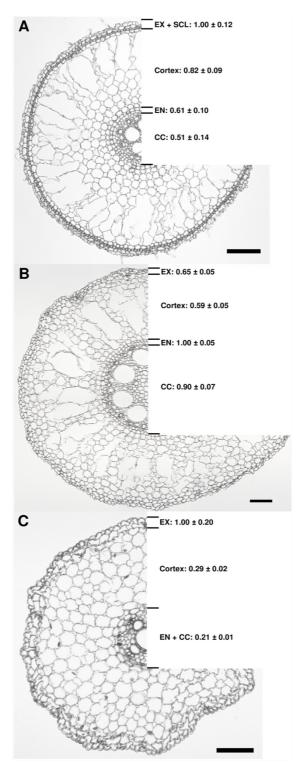


Fig 6. Distribution of Si in the root zone B of rice (A), maize (B) and onion (C). The values indicate the relative Si abundance relating to a maximum value of 1.0. Root zone B was at 4–6 cm drt in rice and onion and at 6–8 cm drt in maize roots. Data are mean  $\pm$  s.e., n=4. The black bar indicates 100  $\mu$ m. EX, exodermis; SCL, sclerenchyma; EN, endodermis; CC, central cylinder. The formation of an aerenchyma was observed not only in rice but also in maize roots, which was reported before for well-aerated maize plants [61].



### Casparian bands

Exodermal CB in rice and onion roots was already initiated in the root tip (0–2 cm), while CB in the maize root exodermis were detected in the root zone at 4–6 cm (Fig 3). This is in line with literature, where the development of exodermal CB was reported at 3 cm drt in rice plants grown in nutrient solution [33] or even at 1 cm drt when grown in stagnant deoxygenated medium [34, 35]. Comparative analysis made by Schreiber et al. [20] revealed that the exodermis was already fully developed at 6 cm drt in rice roots, but was not yet complete at 12 cm in maize roots. Onion plants grown either in vermiculite or in nutrient solution showed CB in the exodermis at 5 cm drt [36, 37].

Generally, the formation of exodermal CB at a short distance from the root tip coincided with a low root growth rate in onion and rice compared to maize (Fig 1C), which is in accordance with studies reporting an earlier development of CB in rice and maize exodermis when root growth was reduced [29, 38, 39]. The formation of CB was enhanced in all plant species when Si was supplied (Fig 3). This confirms previous observations in rice [11], while it was reported for the first time for maize and onion. Si also induced suberin lamaellae formation (S5 Fig). The simultaneous development of CB and suberin lamaellae in the exodermis agrees with literature [47]. This Si effect did not result from slowed root growth, since root growth rates were not different between Si treatments.

Similar Si effects on CB formation in the exodermis were also observed in the *commelinoid* monocotyledon *Tradescantia virginiana* and in the dicotyledon *Guizotia abyssinica* (S3 and S4 Figs). It was shown in a previous study that the Si-induced CB formation reduced the radial oxygen loss of the rice root [11], thus providing a physiological proof of the Si impact. It was observed in other studies that Si reduced the translocation of sodium and chloride from root to shoot in rice plants, and this was correlated with a reduced apoplastic bypass flow along the root [29, 40, 41]. The latter was suggested to be the result of silica deposition at the endodermis or exodermis. However, the limited bypass flow can also be explained by an enhanced formation of exodermal CB after Si treatment.

Besides the earlier induction of CB formation by Si in roots of all species, the accumulation of Si in the different tissues correlates with the occurrence of exodermal apoplastic barriers. In rice and onion, where the apoplastic pathway for water and solutes is limited by the early formation of exodermal CB in root zone B (Fig 3A and 3C), Si accumulates at this border tissue (Fig 6A and 6C), whereas in maize, where CB develop much later in the exodermis (Fig 3B), the highest Si concentration is located at the endodermis (Fig 6B). This accumulation probably increases the promotional effect on CB formation.

### Suberin and lignin

A promoted formation of CB would suggest increased amounts of its potential components suberin and lignin, but surprisingly this was not found. By contrast, Si did not affect the suberin in the outer part of the onion root and even decreased the total suberin content in the outer cell layers of rice and maize root (Fig 4A, 4B and 4C). This decrease was due to a reduced quantity of the aromatic suberin, while the aliphatic suberin fraction in all plants was unaffected by Si supply. Si did not affect suberin in onion roots probably because the phenolic suberin fraction was only 13% of the total suberin in onion, while it was 76% and 83% in maize and rice, respectively. Moreover, the main components of the phenolic suberin in rice and maize roots were ferulic acid and p-coumaric acid, which is in line with literature [20, 34, 35, 42]. By contrast, the phenolic suberin in the outer part of the onion roots consisted only of ferulic acid, and lacked p-coumaric acid, which is in accordance with a previous study [43]. The amounts of the phenolic and aliphatic suberin in the outer part of the rice and maize root were in the same



order of magnitude as reported in literature [20, 34, 35] and the amount of aliphatic suberin in the outer part of the onion root was also in similar values to the literature [44]. The formation of exodermal CB along the maturing onion root is accompanied by an increase of the insoluble aliphatic suberin [43]. In our study, this positive correlation between CB development and aliphatic suberin was not found. However, we observed microscopically a simultaneous development of CB and suberin lamaellae (S5). It is assumed that the fluorescent dye fluorol yellow is a much more sensitive indicator of suberin lamellae initiation than the chemical analysis.

The composition of the aliphatic suberin fraction was different between rice, maize and onion roots and was less complex in onion than in rice and maize (S1 Fig). The group of  $\omega$ -hydroxy fatty acids was the most abundant substance class of the aliphatic suberin in the outer part of rice, maize and onion roots, which is congruent with previous studies [20, 35, 42, 44]. The lignin and lignin-like polymer concentration in the outer part of the root was not affected by the Si treatments in any plants (Fig 5). The concentration in rice was twice as high as in maize and onion, probably as a result of the phenol-rich sclerenchyma. The concentration of lignin when expressed on a dry weight basis (S2 Fig) was in the range reported for onion, maize and rice roots [34, 35, 44, 45].

The composition of CB is controversially discussed in literature and it is thought that suberin and lignin are the main components [46, 47]. By contrast, Naseer et al. [48] concluded from studies with *Arabidopsis thaliana* that lignin is indispensable for fully functional CB while suberin is not. On the other hand, lignin was found along with suberin in endodermal CB of several other plants including monocotyledonous and dicotyledonous species [21, 49, 50]. However, Geldner [51] argued that the suberin found is not essential for the formation or part of the CB but rather deposited as suberin lamellae during the second stage of endodermal differentiation.

Independent of the question whether suberin is an integral part of the CB, it was surprising to see that Si, on the one hand, promoted the formation of CB in rice, maize and onion exodermis, but, on the other hand, did not affect or rather decreased the amount of suberin, while the total phenol or lignin concentration was not affected. How can this paradox be explained?

### A model of Si and phenol interaction

The Si-enhanced formation of CB in the exodermis was proven both by microscopical and physiological results and data of the suberin and lignin analysis need to be interpreted. Prior to the suberin analysis, the suberin polymer is depolymerized to its monomers in a transesterification reaction with MeOH/BF<sub>3</sub> [44, 52]. This reaction converts acids that are esterified in a polymer into their respective methylesters [21]. However, monomers connected by bonds other than ester bonds are not cleaved by this method and thus, these monomers are not released and cannot be detected by GC analysis. On the other hand, the lignin analysis is based on a depolymerization reaction using acetyl bromide/acetic acid, which is much more aggressive than the MeOH/BF<sub>3</sub> method and allows complete dissolution of lignin [22]. Other phenolic depolymerization methods resulting in complete dissolution of lignin and lignin-like polymers, such as nitrobenzene oxidation [53] or cupric oxide oxidation [54], would not allow deeper insights since Si did not affect the concentration of lignin and lignin-like polymers, as determined by the acetyl bromide/acetic acid assay. If Si-complexed phenols form non-ester bonds, the amount of detectable aromatic suberin monomers, using the MeOH/BF<sub>3</sub> assay, would be decreased by Si, while the amount of lignin as detected by the acetyl bromide/acetic acid assay would not be affected.

An increase of the lignin and lignin-like polymers due to Si supply could not be observed although the histochemical examination of exodermal CB indicated an increase of this fraction



since the dye berberine-aniline blue has a major affinity for phenolic compounds [17]. This effect revealed by microscopy was presumably not observed in the analytic results since the phenolics contained in the stained fraction were a magnitude lower than total lignin and lignin-like polymers in the same root section (Figs 4 and 5).

An interaction of Si and phenols was discussed several times in literature. It was reported in the early-1970s that silica gel or colloidal silica can form complexes with catechol as six-coordinated Si [55, 56]. Inanaga and Okasaka [57] proposed that Si was associated with phenols in root cell walls of rice and could lead to crosslinking between lignin and carbohydrates. Support for this hypothesis was yielded from *in vitro* studies, where lignin isolated from rice induced the deposition of silica [58]. Moreover, callose was reported to induce silica deposition [59], which is in line with data from the Si-accumulator horsetail (*Equisetum arvense*), where Si was found along with callose in the cell walls [60].

Considering the chemical reactivity of Si with phenols, the accumulation of Si in the exodermis and the promotion of the CB in the exodermis, we hypothesize that Si either crosslinks phenols with the cell wall or induces precipitation of the phenols leading to an enhanced development of CB. This hypothesis considers that Si accumulated in phenol-rich compartments, such as the exodermis and endodermis, and that Si reduced the amount of esterified phenols as determined after depolymerization using the MeOH/BF<sub>3</sub> method whereas the total phenol (esterified and non-esterified complexed phenols) concentration as detected with the lignin determination method was not affected.

In conclusion, we demonstrated that Si enhanced the formation of exodermal CB not only in roots of rice, but also in maize and onion plants as well as in nug and *Tradescantia*. This promotion was not due to increased suberin amounts in the outer part of the roots. Instead, we observed a decrease of esterified phenolic compounds in rice and maize and we suggest that Si induced enhancement of CB might be the result of a chemical interaction of phenolic compounds with Si. The data presented and literature reports provide solid ground for the hypothesized Si mechanism enhancing CB in the exodermis.

### Supporting Information

S1 Fig. Amounts of aliphatic suberin compounds in the outer cell layers of rice, maize and onion roots.

(PDF)

S2 Fig. Amount of the lignin and lignin-like polymers in the outer cell layers of rice, maize and onion roots.

(PDF)

S3 Fig. Development of Casparian bands (CB) in the exodermis of nug plants as affected by Si supply.

(PDF)

S4 Fig. Development of Casparian bands (CB) in the exodermis of *Tradescantia virginiana* plants as affected by Si supply.

(PDF)

S5 Fig. Formation of Casparian bands and suberin lamellae in the exodermis of rice, maize and onion roots as affected by Si supply.

(PDF)



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#### **Author Contributions**

Conceived and designed the experiments: ATF MKS LS. Performed the experiments: ATF SS MH AS FW. Analyzed the data: ATF SS MH AS FW. Contributed reagents/materials/analysis tools: AS FW LS MH. Wrote the paper: ATF MH MKS.

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Figure S1. Amounts of aliphatic suberin compounds in the outer cell layers of rice (A), maize (B) and onion (C) roots.

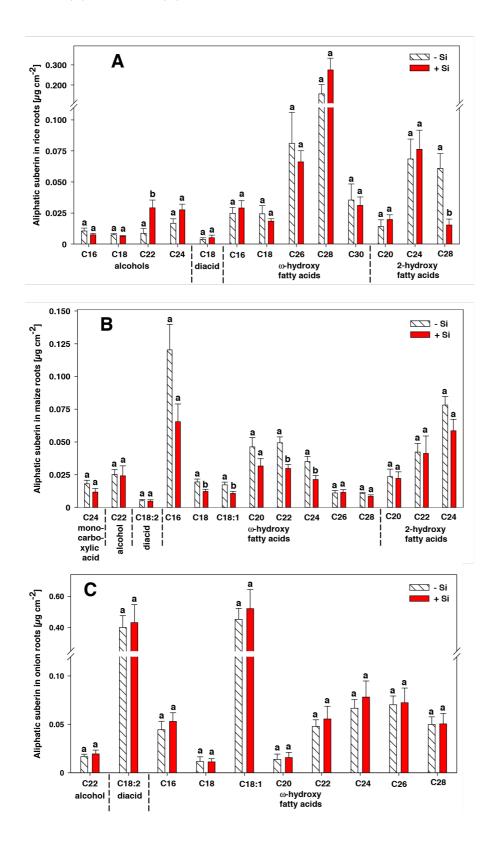


Figure S2. Amount of the lignin and lignin-like polymers in the outer cell layers of rice, maize and onion roots.

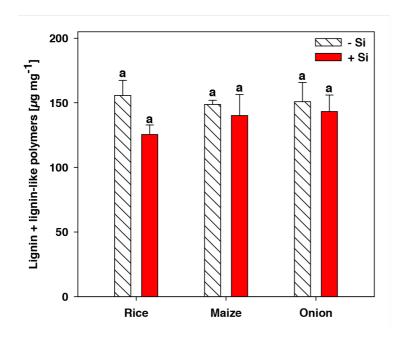


Figure S4. Development of Casparian bands (CB) in the exodermis of *Tradescantia* virginiana plants as affected by Si supply.

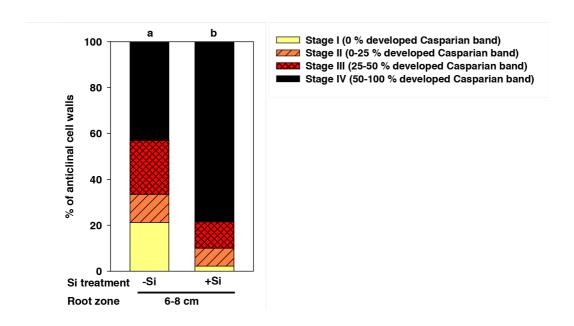
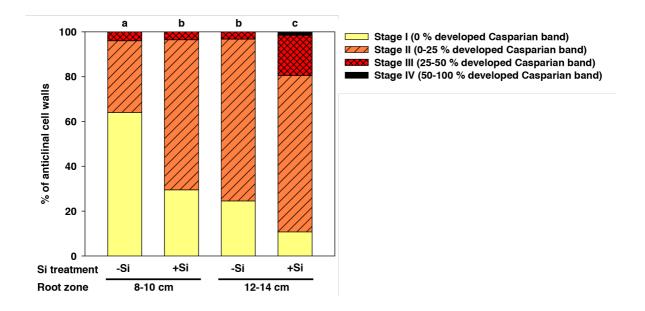


Figure S3. Development of Casparian bands (CB) in the exodermis of nug plants as affected by Si supply.



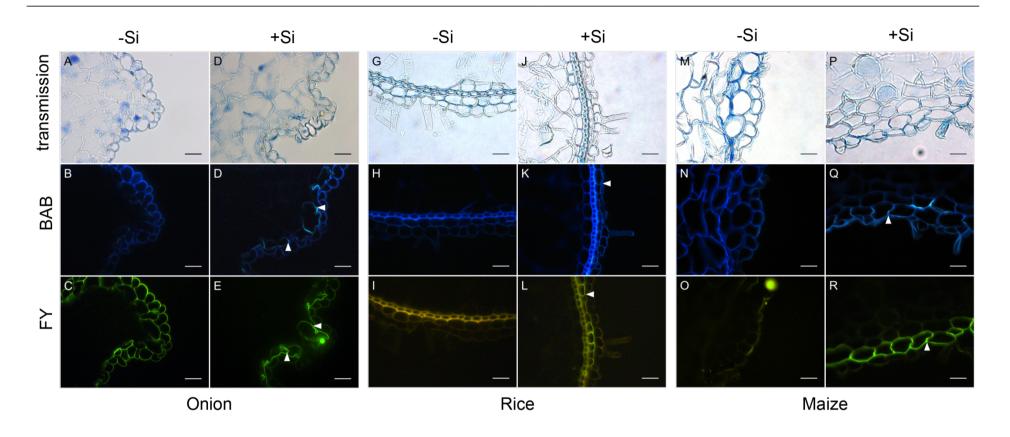


Figure S5: Formation of Casparian bands and suberin lamellae stained with berberine-aniline blue and fluorol yellow, respectively, in the exodermis of rice, maize and onion roots as affected by Si supply. Serial sections of the same root were stained with berberine-aniline blue (BAB: B,E,H,K,N,Q) or fluorol yellow 077 (FY: C,F,I,L,O,R), the range of 4 cm behind the root tip is shown for onion (A-F) and 6 cm for rice (G-L) and maize (M-R). Arrows indicate formation of CB or suberin lamellae. Bar: 25 μm.

## **Chapter II**

# An ABC Transporter Is Involved in the Silicon-Induced Formation of Casparian Bands in the Exodermis of Rice

Martin Hinrichs <sup>1†</sup>, Alexander T. Fleck<sup>1†</sup>, Eline Biedermann<sup>1</sup>, Ngoc S. Ngo<sup>1</sup>, Lukas Schreiber<sup>2</sup> and Manfred K. Schenk<sup>1</sup>

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#### **Contributor Roles:**

<u>Martin Hinrichs</u>: Conceived and designed the experiments; performed the experiments; analyzed the data; contributed reagents/materials/analysis tools; wrote the manuscript.

<u>Alexander T. Fleck:</u> Conceived and designed the experiments; performed the experiments; analyzed the data; contributed reagents/materials/analysis tools;

<u>Eline Biedermann:</u> Performed the experiments; analyzed the data; contributed reagents/materials/analysis tools

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<u>Lukas Schreiber:</u> Conceived and designed the experiments; contributed reagents/materials/analysis tools.

<u>Manfred K. Schenk:</u> Conceived and designed the experiments; performed the experiments; wrote the manuscript.

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# An ABC Transporter Is Involved in the Silicon-Induced Formation of Casparian Bands in the Exodermis of Rice

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Hinrichs M, Fleck AT, Biedermann E, Ngo NS, Schreiber L and Schenk MK (2017) An ABC Transporter Is Involved in the Silicon-Induced Formation of Casparian Bands in the Exodermis of Rice. Front. Plant Sci. 8:671. doi: 10.3389/fpls.2017.00671 Silicon (Si) promotes the formation of Casparian bands (CB) in rice and reduces radial oxygen loss (ROL). Further transcriptomic approaches revealed several candidate genes involved in the Si-induced formation of CB such as ATP binding cassette (ABC) transporter, Class III peroxidases, ligases and transferases. Investigation of these genes by means of overexpression (OE) and knockout (KO) mutants revealed the contribution of the ABC transporter (OsABCG25) to CB formation in the exodermis, which was also reflected in the expression of other OsABCG25 in the Si-promoted formation of CB genes related to the phenylpropanoid pathway, such as phenylalanine-ammonialyase, diacylglycerol O-acyltransferase and 4-coumarate-CoA ligase. Differential CB development in mutants and Si supply also affected the barrier function of the exodermis. OE of the ABC transporter and Si supply reduced the ROL from roots and Fe uptake. No effect on ROL and Fe uptake could be observed for the KO mutant. The presented research confirms the impact of the OsABCG25 in the Si-promoted formation of CB and its barrier functions.

Keywords: radial oxygen loss, Fe uptake, phenylpropanoid metabolism, CaMV 35s enhancer trap lines, LRR, bypass flow

#### INTRODUCTION

Silicon (Si) is not an essential element, but has several beneficial effects on plant growth. It is one of the most abundant elements in the soil surface, with a soil solution concentration of 2.5–20 mg Si\*L<sup>-1</sup> silicic acid (Bogdan and Schenk, 2008; Marxen et al., 2016). Plants differ in their ability to accumulate Si and rice is known to be a strong accumulator, containing Si in even higher concentrations than nitrogen, potassium or calcium (Epstein, 1994). One of the Si effects is the formation of mechanical barriers in leaves and roots (Cai et al., 2009). Rice is cultivated under flooded (anaerobic) and unflooded (aerobic) conditions. Under submerged conditions adventitious rice roots develop aerenchyma by the lysis of cell walls in the cortex to ensure oxygen supply to the root tips from the shoot (Nishiuchi et al., 2012). However, oxygen diffuses from the aerenchyma into the rhizosphere which is hampered by the Casparian band (CB) in the exodermis. The CB development starts about 5–8 cm behind the root tip in the anticlinal cell walls and is mostly completed at a 12–13 cm distance from the root tip, reducing the radial oxygen loss (ROL) from roots (Steudle, 2000; Vaculík et al., 2009; Fleck et al., 2011). The CBs in the exodermis are also

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thought to reduce the inflow of freely available ions from the soil solution into the cortex (Yeo et al., 1999; Gong et al., 2006; Faiyue et al., 2010). The CB development in the exodermis is stimulated by the Si supply in rice and also in other species, such as *Zea mais*, *Allium cepa*, *Tradescantia virginiana*, and *Guizotia abyssinica*, restricting the ROL to the first 5 cm behind the root tip in rice (Fleck et al., 2015). Furthermore, it was shown that Si supply reduces the Fe concentration in shoot matter (Ma and Takahashi, 1990; Dufey et al., 2013). It is hypothesized that Sistimulated CB formation reduces the Fe flow into the cortex, where it is bound to Deoxymugineic acid and taken up via yellow stripe- like transporters (Yehuda et al., 1996; Nozoye et al., 2011).

The CBs occur in the endodermis of all species and in the exodermis of most species, including rice, maize and onion, but not in A. thaliana (Schreiber et al., 1999; Ma and Peterson, 2003; Naseer et al., 2012; Fleck et al., 2015). The main components are lignin and suberin (Schreiber et al., 2005b; Naseer et al., 2012). Suberin is a biopolymer consisting of aliphatic components (ω-hydroxy acids, α, ω-dicarboxylic acids, fatty acids, alcohols) and aromatic components (ferulic acid; Franke et al., 2005). The CB formation in the endodermis of A. thaliana starts with the deposition of lignin hampering the flux from the cortex into the stele. In the second step, suberin is deposited (Naseer et al., 2012). The CB in the exodermis of rice is formed by simultaneous incorporation of lignin and suberin into anticlinal cell walls (Kotula et al., 2009; Fleck et al., 2015). These CB compounds are provided by the phenylpropanoid pathway and it has been shown that some of the genes, like phenylalanine-ammonia-lyase (PAL), 4-coumarate-CoA ligase (4CL), glycerol-3-phosphate acyltransferase (AT), diacylglycerol O-acyltransferase (DGOAT), ATP binding cassette (ABC) transporter and class III peroxidases (POD) involved in this secondary metabolism pathway are upregulated through Si in roots (Fleck et al., 2011). Additionally, expression of a leucine-rich repeat (LRR) family protein and an ABC transporter (OsABCG25; LOC\_Os10g30610) was enhanced. The transporter OsABCG25 was suggested to be involved in the transport of monolignols or suberin monomers in the Si-induced development of CB in the exodermis. The PAL desaminates phenylalanine to cinnamic acid, which is metabolized via several steps to precursors of lignin and suberin (Zhong et al., 1998; Eckardt, 2002). The 4CL catalyzes the formation of monolignols from coumaroyl-CoA, feruloyl-CoA, or sinapoyl-CoA. Suberin consists of aliphatic and aromatic components where the aliphatic components are provided from fatty acids by POD. The aliphatic and aromatic components, are bound to glycerol by AT, such as DGOAT to suberin monomers. Both monolignols and suberin monomers are most probably transported by ABC transporters into the apoplast (Landgraf et al., 2014; Shiono et al., 2014). The function of these genes in Si-stimulated CB development was studied in overexpression (OE) and knockout (KO) mutants. We observed the involvement of the OsABCG25 in CB formation and, additionally, investigated the expression of genes related to lignin and suberin metabolism. Furthermore, the barrier function of exodermal CB with regarding ROL from roots and Fe uptake were investigated in these mutants.

#### **MATERIALS AND METHODS**

#### **Selection of Rice Mutant Lines**

We selected 24 rice mutant lines carrying a T-DNA insertion that contains multimerized cauliflower mosaic virus (CaMV) 35S enhancers leading to an OE of nearby genes (Jeong et al., 2002; Chern et al., 2007). As selection criteria, the T-DNA insertion of the mutant lines should be located within 10,000 bp upstream or downstream of one of eight candidate gene for suberin or lignin synthesis and must not interrupt the sequence of a nontarget gene. In contrast to the other lines, the line 1B-14436 carried an insertion in the exon sequence of a candidate gene, resulting in an interrupted transcription of the gene OsABCG25 (LOC\_Os10g30610; Genebank ID: ABB47708.1; Uniprot ID: B9G5Y5). The positions of the T-DNA relative to the target gene were calculated using the GenomeBrowser of the OryGenesDB database<sup>1</sup> (Droc et al., 2006). The mutant lines selected were ordered from the Pohang University of Science and Technology (Postech; Pohang, Republic of Korea; Jeon et al., 2000) and from the Taiwan Rice Insertional Mutants Database (TRIM; Taiwan). Table 1 summarizes the mutant lines, the target gene identifiers, the position of the insert relative to the start codon of the target gene, and the supplier of the seeds. Out of these 24 rice mutant lines a total of ten homozygous lines were obtained.

<sup>1</sup>http://orygenesdb.cirad.fr/

TABLE 1 | Mutant lines, target genes, position of the insert relative to the gene and supplier of the seeds.

| Mutant line | Target gene    | Position relative to<br>start codon of gene | Supplier |
|-------------|----------------|---|----------|
| 1B-04415    | LOC_Os01g67540 | 245 upstream                                | Postech  |
| 3A-14487    | LOC_Os01g67540 | 759 upstream                                | Postech  |
| 2D-41110    | LOC_Os02g41680 | 5270 downstream                             | Postech  |
| M0060856    | LOC_Os02g41680 | 59 downstream                               | TRIM     |
| 4A-50856    | LOC_Os05g20100 | 161 downstream                              | Postech  |
| 5A-00450    | LOC_Os06g16350 | 3745 upstream                               | Postech  |
| 5A-00464    | LOC_Os06g16350 | 3820 upstream                               | Postech  |
| 3A-01911    | LOC_Os06g16350 | 10457 upstream                              | Postech  |
| M0038578    | LOC_Os06g22080 | 3644 downstream                             | TRIM     |
| 3D-01082    | LOC_Os06g22080 | 9232 downstream                             | Postech  |
| 3A-01215    | LOC_Os08g02110 | 392 downstream                              | Postech  |
| 3A-02897    | LOC_Os08g02110 | 420 upstream                                | Postech  |
| 3A-08589    | LOC_Os08g02110 | 9509 upstream                               | Postech  |
| 3A-06124    | LOC_Os10g30610 | 1466 upstream                               | Postech  |
| 3A-16329    | LOC_Os10g30610 | 3131 upstream                               | Postech  |
| 3A-16331    | LOC_Os10g30610 | 3874 upstream                               | Postech  |
| 3A-02127    | LOC_Os10g30610 | 4537 upstream                               | Postech  |
| 3A-60593    | LOC_Os10g30610 | 4139 upstream                               | Postech  |
| 2D-00893    | LOC_Os10g30610 | 5277 downstream                             | Postech  |
| M0033740    | LOC_Os11g14050 | 7665 upstream                               | TRIM     |
| M0058854    | LOC_Os11g14050 | 3592 downstream                             | TRIM     |
| 2A-20141    | LOC_Os11g14050 | 1810 downstream                             | Postech  |
| M0066685    | LOC_Os11g14050 | 12599 downstream                            | TRIM     |
| 1B-14436    | LOC_Os10g30610 | Exon  | Postech  |

# Plant Material, Growth Conditions and Harvest, T1 Seeds

Rice (*Oryza sativa* L.) seeds of the insertion lines (**Table 1**) were delivered as T1 seeds containing a mixture of wild type (WT), heterozygous and homozygous mutant plants. Seeds were germinated in tap water for several days for seed propagation and the seedlings were then transferred to 10-L pots containing soil from the local campus and grown submerged in a greenhouse with average temperatures around 28°C and a minimum of 220  $\mu$ mol  $M^{-2}$  s $^{-1}$  light intensity until maturity. After a few weeks, the genotype of the plants was determined using DNA from the leaves and only homozygous mutant plants were further cultivated further. Whole plants were individually enwrapped in plastic sleeves at the time of flowering to prevent crosspollination with other plants.

#### Genotyping

The DNA extraction was performed using a crude leaf extract from a NaOH-Tris-extraction method for genotyping (Collard et al., 2007). An amount of 200 mg of a leaf were harvested, transferred to a 2.0-ml tube containing one steel ball and 100  $\mu$ l of 0.5 M NaOH. The leaf was homogenized in a swing mill for 1 min at 30 Hz and then 900  $\mu$ l of 0.1 M Tris was added. Samples were centrifuged for 3 min at 13000  $\times$  g, the supernatant was transferred to a fresh 1.5-ml tube and stored at  $-20^{\circ}$ C.

An amount of 1 µl of the DNA extracted was used in 25 µl PCR reaction mix containing 2.5 µl 10x reaction buffer, 3.6 mM MgCl<sub>2</sub>, 0.2 mM dNTPs (Fermentas, St. Leon-Rot, Germany), 0.75 U Taq-DNA-polymerase (DNA cloning service, Hamburg, Germany), 0.25 μM forward primer and 0.25 μM reverse primer. Two PCR runs with different primer combinations were used for each insertion line. A primer pair targeting at genomic regions flanking the insert was used (W-primer pair) in the first PCR, and an insert-specific primer targeting at a sequence near the border of the T-DNA was used together with one primer of the first PCR (I-primer pair) in the second PCR. The PCR products were electrophoretically separated on a 1% agarose gel. The WT plants showed a band with the W-primer pair, homozygous mutant plants were identified by a band with the I-primer pair, while heterozygous plants were characterized by bands with both primer pairs. The PCR runs included both negative controls with water instead of DNA and positive controls with DNA from WT plants. The genotyping primers used for each line are summarized in Supplementary Tables S1, S2.

# **Growth Conditions and Harvest, T2 Seeds**

The T2 seeds of homozygous mutant plants and corresponding WT plants were germinated in tap water for several days and then placed between two layers of filter paper standing in tap water for 7 days. Seedlings were transferred to nutrient solution in 5-L pots containing 0.43 mM NH<sub>4</sub>NO<sub>3</sub>, 0.32 mM NaH<sub>2</sub>PO<sub>4</sub>, 0.51 mM K<sub>2</sub>SO<sub>4</sub>, 1 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 1.6 mM MgSO<sub>4</sub>, 1.82  $\mu$ M MnSO<sub>4</sub>, 0.03  $\mu$ M (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>, 9 H<sub>3</sub>BO<sub>3</sub>, 0.6  $\mu$ M ZnSO<sub>4</sub>, 0.15  $\mu$ M CuSO<sub>4</sub>, and 35.81  $\mu$ M Fe<sup>EDDHA</sup>. The pH value was adjusted to 6.0 by the addition of 10% H<sub>2</sub>SO<sub>4</sub> and 1 M KOH and the

nutrient solution was renewed weekly for the first 2 weeks. After 2 weeks, the nutrient solution was changed twice a week until harvest. Plants were cultivated in a climate chamber (photoperiod 14/10 h light/dark; temperature,  $25/20^{\circ}\text{C}$  day/night; 75% relative humidity and a light intensity of  $220 \mu \text{mol m}^{-2} \text{ s}^{-1}$ ).

The WT seeds and T2 seeds of homozygous mutant plants of the lines 1B-14436 (KO) and 3A-16329 (OE) were germinated and cultivated in nutrient solution as described above, but with two Si treatments, to determine the effect of Si supply. Si was applied as silica gel and Si concentrations were 3 or 30 mg L $^{-1}$  resulting in Si concentrations of 0.1/0.1 and 60/3 mg $^{*}$ g $^{-1}$  shoot / root DM, respectively (Supplementary Figure S1).

The homozygous genotype of the plants was confirmed during the cultivation using DNA from the leaves. After 28 days in nutrient solution, root zones 4–6 cm behind the root tip were harvested and either stored in 70% ethanol at  $4^{\circ}\mathrm{C}$  for subsequent histochemical determination of CB or transferred immediately to liquid nitrogen and stored at  $-80^{\circ}\mathrm{C}$  for transcript analysis. Shoot and root were separated, dried at  $60^{\circ}\mathrm{C}$  for 4 days and weighed.

#### **Histochemical Examination of Roots**

Freehand cross sections of adventitious roots fixed in 70% EtOH were stained with 0.1% (w/v) berberine hemisulfate for 60 min, washed three times with distilled water and counterstained with 0.5% (w/v) aniline blue for a further 30 min for detection of CB (Brundrett et al., 1988). Stained sections were mounted in 0.1% (w/v) FeCl<sub>3</sub> in 50% (v/v) glycerine and examined using an Axioskop fluorescence microscope (Zeiss, Jena, Germany) with UV illumination and excitation filter G 365, chromatic beam splitter FT 395 and barrier filter LP 420. Pictures were taken with the AxioCam MRc (Zeiss) and picture recording software (AxioVision Ac, Version 4.4, Zeiss). Suberin exhibited a bluewhite color under UV light. The development of CB in the anticlinal exodermal cell walls was determined and allocated to one of four stages: 0% (stage I), 0-25% (II), 25-50% (III) and 50-100% (IV) development of CB in the anticlinal cell wall of the exodermis.

Five roots without lateral roots were taken from each of the four replicates for cross-sectioning and 20 cells each from five cross sections were used for microscopic examination, therefore, the degree of development of CB was based on 400 cell walls per treatment.

#### Transcript Analysis

Frozen root material was ground under liquid nitrogen and total RNA was isolated using TRIsure® Reagent (Bioline, Luckenwalde, Germany), following the manufacturer's RNA instructions. The determined quality was electrophoretically by 2% non-denaturating agarose gel and fluoretically using a Nanophotometer (Implen, Munich, Germany). The total RNA (1  $\mu g)$  and random hexamer primers were used to synthesize first-strand cDNA using the Revert Aid<sup>TM</sup> H Minus Kit (Fermentas, St. Leon-Rot, Germany), following the manufacturer's instructions for GC-rich templates.

In the qRT-PCR experiments, 100 ng cDNA was used as a template in 25 µl reaction mix containing 2.5 µl 10x buffer,

3.6 mM MgCl<sub>2</sub>, 0.2 mM dNTPs mix (Fermentas, St. Leon-Rot, Germany), 0.25  $\mu l$  1:1000 diluted SYBR-Green (Invitrogen, Carlsbad, CA, USA), 0.75 U HotStart-Taq-DNA-Polymerase (DNA cloning service, Hamburg, Germany), and 0.25  $\mu M$  forward and 0.25  $\mu M$  reverse primers. The qRT-PCR runs were performed in the CFX96 cycler (Bio-Rad, München, Germany), using an initial 95°C-step for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 30 s and 72°C for 30 s, and a final melting curve procedure with a stepwise increment of 1°C ranging from 60 to 95°C.

The eukaryotic elongation factor 1-alpha (eF1- $\alpha$ ) was used as an endogenous control due to its stable transcript abundance in rice (Jain, 2009). A list of primer sequences used can be found in Supplementary Table S3. Three technical and three biological replicates were used for each target in qRT-PCR. The relative quantity was calculated using the R-Macro "qpcrmix" (Steibel et al., 2009), based on the  $2^{-\Delta\Delta CT}$  method.

# **Cell Wall Isolation and Preparation for Suberin Analysis**

Root zones 4-6 cm behind the root tip were harvested and the root surface was scanned using WinRHIZO software (Regent Instruments Inc., Quebec, QC, Canada). The cell wall isolation and preparation was performed as described in detail by Schreiber et al. (1994). Briefly, root zones were washed with H<sub>2</sub>O<sub>dest</sub> and then incubated at room temperature for 4 days in 1 ml enzyme solution (0.1 M citric acid monohydrate, 1% pectinase (v/v), 1% cellulase (v/v), 0.1% NaN<sub>3</sub>), which was renewed daily. After enzymatic digestion, the non-degradable outer part of the root comprising the exodermal cell wall fraction and the sclerenchyma was separated from the tissue containing the stele by using two forceps under a binocular. The exodermal cell wall material was incubated in enzyme solution for another 2 days to remove any residual cortex material. Subsequently, the isolates were washed with H2Odest and incubated in borate buffer (0.01 M sodium borate, pH 9) for 2 days.

Dried isolated cell wall material was extracted for 5 days with a 1:1 mixture of chloroform and methanol, which was changed daily. After the extraction, the isolated samples were dried for 2 h in the desiccator over silica gel. The dry weight was determined just prior to the suberin analysis of isolated samples.

#### Suberin Analysis

The dried sample isolates were incubated for 16 h in 1 N methanolic boron trifluoride (MeOH/BF3; Fluka/Sigma-Aldrich, St. Louis, MO, USA) at 70°C for transesterification. Saturated NaOH was added to stop the transesterification reaction and to advance the following phase separation. Dotriacontan ( $C_{32}$  alkane, 10.025 mg/ 50 ml) was added to each sample as an internal standard. The soluble hydrophobic components were extracted by adding chloroform. The chloroform phase was transferred to a new vial and extraction was repeated three times. The extract was dried with water-free Na<sub>2</sub>SO<sub>4</sub> and the volume was reduced to 50  $\mu$ l by evaporation under N<sub>2</sub> flow.

Samples were derivatized in 20 µl BSTFA (N,N-bis(trimethylsilyl)-trifluoracetamide; Machery-Nagel, Düren,

Germany) and 20  $\mu$ l dry pyridine (GC-grade, Merck, Darmstadt, Germany) for 40 min at 70°C. Pyridine catalyzed the derivatization reaction and BSTFA masked free hydroxyland carboxyl-groups forming the corresponding trimethylsilyl derivatives (Schreiber et al., 2005b). Samples were analyzed by gas chromatography (GC; Type: 6890N, Agilent Technologies, Santa Clara, CA, USA) and mass spectroscopy (MS: Type: 5973N, Agilent Technologies, Santa Clara, CA, USA). The GC and MS analyses were performed as described previously in detail in Zeier and Schreiber (1997). The quantification of the monomers was performed using a gas chromatograph combined with a flame ionization detector. Four replicates of each rice line were used

#### Visualization of Radial Oxygen Loss

In order to visualize the ROL, adventitious roots of plants were grown for 28 days in nutrient solution with and without Si supply, as described above. An adventitious root was placed between two acryl glass plates (16 cm × 6 cm; 0.5 cm apart) which were sealed with Plasticine (Pelikan, Hannover, Germany) and the rest of the root system remained in nutrient solution. The space between the plates with the root was filled with 38°C warm semisolid agar medium containing FeS by use of a pipette and the top was sealed with paraffin wax. The medium was prepared by adding 0.8% agar to iron-free nutrient solution and subsequent heating to solubilize the agar. The solution was amended with 1.4 g  $\text{FeSO}_4 \times 7~\text{H}_2\text{O}~\text{L}^{-1}$  and  $0.32~\text{g}~\text{Na}_2\text{S}~\text{L}^{-1}$  , whereupon a black FeS precipitation developed (Trolldenier, 1988). Finally, the solution was buffered by the addition of 0.5 g CaCO<sub>3</sub> L<sup>-1</sup> and adjusted to pH 6.0. The acryl glass plates, held together by clamps, and the plant were fixed using a tripod. The plates were covered with aluminum foil and scans of the plates were taken by a flatbed scanner (Expression 1600, Seiko Epson K.K., Suwa, Nagano, Japan). 12 h after embedding in agar, six roots were investigated for each treatment. The area of ROL was determined using the Fiji imaging software (Schindelin et al., 2012), transforming the picture into a binary image and analyzing particles bigger than 50 pixels.

#### **Chemical Analysis**

In order to determine the Si concentration in the shoot and root, 200 mg dried and ground plant matter was digested in 3 ml 65% HNO<sub>3</sub>, 2 ml  $\rm H_2O$  and 2 ml 30%  $\rm H_2O_2$  in a microwave for 12 min at 190°C, then diluted with 20 ml 10% NaOH, neutralized with HNO<sub>3</sub> (Haysom and Ostatek-Boczynski, 2006) and filled up to a final volume of 100 ml.

In order to determine the Fe, Cu, Mn and K, 50 mg of dried and ground shoot matter was digested in 2 ml 65%  $HNO_3$ , 2 ml  $H_2O$ , and 0.5 ml 30%  $H_2O_2$  in a microwave for 25 min at 190°C and then diluted with distilled water to 25 ml.

The Si, Fe, Cu, Mn, and K in the plant extracts and nutrient solution were determined by ICP-MS (7500c Agilent Technologies, Santa Clara, CA, USA).

#### **Statistical Analysis**

All treatments were replicated four times unless stated otherwise and the mean of the treatments were compared using *t*-test,

Tukey or Bonferroni test using Sigma Plot (Systat Software Inc., San Jose, CA, USA). A cumulative link mixed model was calculated with p < 0.05 using the package ordinal in R Software (R Development Core Team, 2011) for comparison of the developmental stages of CB. The statistical qRT-PCR analysis was performed using the R-Macro of Steibel et al. (2009).

#### **RESULTS**

A total of ten homozygous rice lines were investigated to observe the function of Si enhanced genes involved in the lignin and suberin synthesis (**Table 2**). Only one KO (1B-14436) and one OE (3A-16329) line showed a significant reduction or increase of the transcript level compared to WT for the ABC transporter *OsABCG25* (LOC\_Os10g30610).

Results given in **Figure 1** confirm down- and upregulation of this gene in the respective mutant at low and high Si supply. The expression in OE plants was increased by a factor of four at both Si levels, whereas the transcript level in KO plants was reduced to 1/6. High Si supply enhanced the gene expression by a factor of 2 in WT and OE plants, but did not in KO plants.

However, there was no pronounced effect of differential gene expression in mutants on suberin fractions in outer cell layers (OPR). The Total content of  $\omega$ -hydroxy fatty acids and 2-hydroxy fatty acids, and fractions of C24 acids, C24 alcohols, C24 diacids,  $\omega$ -hydroxy fatty acids and 2-hydroxy fatty acids were not significantly affected (Supplementary Figures S2, S3). The content of aromatic compounds, both coumaric acid and ferulic acid, in the OPR was decreased in the KO mutants, but the OE mutants did not differ clearly from the WT plants (Supplementary Figure S4).

The downregulation of one gene involved in CB development should result in an altered regulation of other genes known to be involved in CB development from previous studies (Fleck et al., 2011). The KO of *OsABCG25* resulted in a downregulation of PAL (LOC\_Os02g41680) and LRR (LOC\_Os11g14050) by 30% (**Figure 2**). The OE resulted in an upregulation of DGOAT

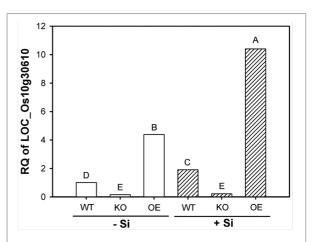


FIGURE 1 | Transcription of the gene LOC\_Os10g30610 (OsABCG25) in root zone 4–6 cm of wild type (WT), knockout (KO) line 1B-14436 and overexpression (OE) line 3A-16329 (OE) plants grown in nutrient solution with low (–Si, 3 mg Si L $^{-1}$ ) and high (+Si, 30 mg L $^{-1}$ ) Si supply. Relative quantity (RQ) of WT –Si = 1. Different letters indicate significant differences between treatments at  $\rho < 0.01$ .

(LOC\_Os06g22080), PAL and LRR by a factor of 1.5 to 2. Additionally, 4CL (LOC\_Os01g67540) was slightly downregulated. Moreover, POD1 and 2 (LOC\_Os08g02110 and LOC\_Os06g16350) and AT (LOC\_Os05g20100) were not regulated.

Furthermore, microscopic evaluation of the exodermis development in berberin-aniline stained hand cuttings of the zone 4–6 cm behind the root tip showed different patterns of CB formation (Figure 3). High Si supply enhanced in WT and the mutants the CB development which was reflected in a decreased number of cell walls without CB and a higher number of cell walls of Stage III or even fully developed CB. Furthermore, the CB formation in the OE mutant compared to the WT was significantly enhanced in both Si levels. However, the KO mutant was not different from the WT.

TABLE 2 | Homozygous rice insertion lines and target genes related to suberin and lignin synthesis, gene loci and changes in target gene transcription relative to wild type (WT) plants.

| Insertion line | Target gene                         | Gene Locus     | Transcription of target gene in<br>insertion line relative to WT |
|----------------|-------------------------------------|----------------|--|
| 2D-41110       | Phenylalanine ammonia-lyase         | LOC_Os02g41680 | 1.44 n.s.  |
| 3A-14487       | 4-coumarate-CoA ligase-like 6       | LOC_Os01g67540 | 0.53 n.s.  |
| 3A-01911       | Class III peroxidase                | LOC_Os06g16350 | 1.83 n.s.  |
| 3A-08589       | Class III peroxidase                | LOC_Os08g02110 | 1.05 n.s.  |
| IB-14436 (KO)  | ABC transporter OsABCG25            | LOC_Os10g30610 | 0.21***  |
| BA-06124       | ABC transporter OsABCG25            | LOC_Os10g30610 | 1.05 n.s.  |
| BA-16329 (OE)  | ABC transporter OSABCG25            | LOC_Os10g30610 | 5.01***  |
| BA-02127       | ABC transporter                     | LOC_Os10g30610 | 1.7 n.s.   |
| A-60593        | ABC transporter                     | LOC_Os10g30610 | 1.36 n.s.  |
| M0066685       | Leucine-rich repeat receptor kinase | LOC_Os11g14050 | 1.78 n.s.  |

n.s, not significant (p > 0.05); \*\*\* significant with p-Value < 0.05.

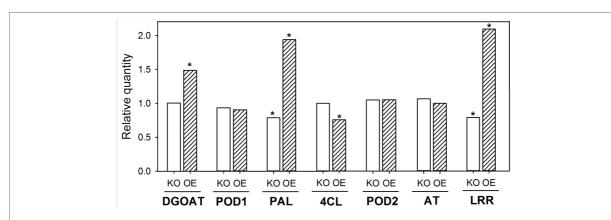


FIGURE 2 | Transcription of Casparian band (CB)-related genes in root zone 4–6 cm of WT, KO line 1B-14436 and OE line 3A-16329. The OE plants were grown in nutrient solution with low (–Si, 3 mg Si L $^{-1}$ ) Si supply and KO grew in high (+Si, 30 mg L $^{-1}$ ) Si supply. The RQ of corresponding WT (–Si /OE; +Si/KO) = 1. Abbreviations of the genes are: 4CL, 4-coumarate ligase (LOC\_Os01g67540); AT, glycerol-3-phosphate acyltransferase (LOC\_Os05g20100); DGOAT, diacylglycerol O-acyltransferase (LOC\_Os06g22080); LRR, leucine-rich repeat family protein (LOC\_Os11g14050); PAL, phenylalanine-ammonia-lyase (LOC\_Os02g41680); POD1 and 2, peroxidase (LOC\_Os08g02110 and LOC\_Os06g16350). A \*indicates significant differences between the WT and mutant plant at  $\rho$  < 0.01.

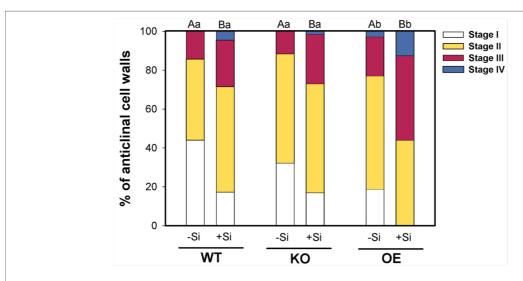


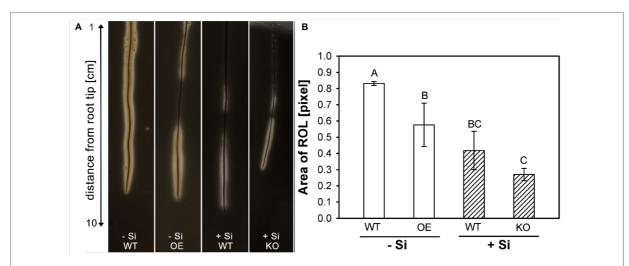
FIGURE 3 | Development of CB in the exodermis of WT plants and the mutant lines 1B-14436 (KO) and 3A-16329 (OE) as affected by Si supply (concentration are given in the legend of Figure 1). The root zone investigated was 4–6 cm behind the root tip. The CB formation was classified in four stages: I:0, II: 0–25, III: 25–50, IV: >50% of the length of the anticlinal cell wall with CB n = 4. Different capital and small letters indicate significant differences between Si treatments within a genotype and for genotypes within the same Si level, respectively; cumulative link mixed models with p < 0.05.

These findings agreed well with the observations on ROL from rice roots (**Figure 4**). Rice roots were embedded in iron-sulfur agar to visualize the ROL as a clear zone along the root. The KO line was investigated under +Si conditions, since a fully developed ROL can be already expected for WT roots under -Si conditions. By contrast, ROL was investigated for the OE line under -Si, since the ROL of WT roots under +Si is already heavily restricted to the 5 cm behind the root tip (Fleck et al., 2011).

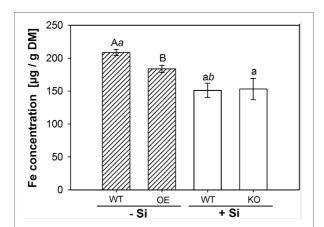
The results confirmed previous reports (Fleck et al., 2011, 2015) that Si reduces the ROL from WT roots. The clear zone

in  $-\mathrm{Si}$  plants, extended along the whole root length and even precipitation of  $\mathrm{Fe^{3+}}$  hydroxide/oxide was visible at the root surface. The area of ROL was reduced by half under high Si and restricted to the first 5 cm behind the root tip. The OE line had a clearly reduced ROL compared to WT, whereas the KO mutant behaved like the WT.

Silicon application was reported to decrease the Fe concentration in rice shoots (Ma and Takahashi, 1990; Dufey et al., 2013). We speculated that this effect might be related to the CB-formation and analyzed the Fe concentration in shoot matter (**Figure 5**). Si application reduced Fe concentration in



**FIGURE 4 | (A)** Visualization of radial oxygen loss (ROL) from rice roots using a FeS-agar. The WT plants were grown with low (–Si) and high (+Si) Si supply (conc. are given in the legend of **Figure 1**), whereas the insertion line 1B-14436 (KO) was cultivated only in + Si and mutant 3A-16329 (OE) only in –Si; **(B)** Area of ROL of roots of WT plants and the mutation lines 1B-14436 (KO) and 3A-16329 (OE). Different letters indicate a significant difference between treatments; Tukey test with  $\rho < 0.05$ .



**FIGURE 5 | Iron content in shoot dry matter.** The WT plants were grown with low (–Si) and high (+Si) Si supply (conc. are given in the legend of **Figure 1**), whereas the insertion line 1B-14436 (KO) was cultivated only in +Si and mutant 3A-16329 (OE) only in –Si. Different capital, small and italic letters indicate a significant difference between treatments; students t-test with p < 0.05.

WT shoots by 20%. A significant reduction compared to the WT under —Si was also observed in the OE mutant, whereas the KO line was not different to the WT under +Si.

#### **DISCUSSION**

#### **Gene Expression**

The KO line and one OE line out of nine homozygous lines showed a significant differential expression of the gene of interest

indicating a very low efficiency of the CaMV 35s promoter enhancer-trap transgenic lines (**Table 2**). The CaMV 35s was described to be less active in monocots, roots and rhizodermal cells (Battraw and Hall, 1990; McElroy et al., 1990). Obviously, CaMV 35s enhancer-trap transgenic lines were not suitable to investigate gene function in rice roots. Additionally, we observed only heterozygous progenies for some CaMV 35s enhancer trap-lines (data not shown). The reasons could be lethality of homozygous plants and/or apomixis (Bicknell and Koltunow, 2004).

The KO and OE of the ABC transporter OsABCG25 (LOC\_Os10g30610) resulted in down- and upregulation of some genes involved in the lignin and suberin metabolism, as summarized in Figure 6 (Figures 1, 2). The PAL (LOC\_Os02g41680), catalyzing the first step of the phenylpropanoid pathway, and the LRR receptor-like kinase (LOC\_Os11g14050) were downregulated in KO, but upregulated in OE. Furthermore, the 4CL (LOC\_Os01g67540) in OE was downregulated and an upregulation of DGOAT (LOC\_Os06g22080) was observed. The 4CL metabolizes p-coumaric acid and ferulic acid to monolignols (Gross and Zenk, 1974). DGOAT is most probably involved in the esterification of glycerol compounds into aliphatic suberin precursors in roots (Cases et al., 1998; Lu et al., 2003), to which ferulic acid is transferred by aliphatic suberin feruloyl transferase (Molina et al., 2009) to generate suberin monomers. These observations suggest that the formation of monolignols in the OE mutant was reduced, whereas the pathway to suberin monomers was enhanced, leading to the speculation that suberin monomers are the substrate of the OE ABC transporter OsABCG25 (Figure 6). This transporter has no sequence similarity to ABCG1, a suberin transporter which has already been described from Arabidopsis thaliana (ABCG1, Landgraf et al., 2014). Furthermore, the ABC

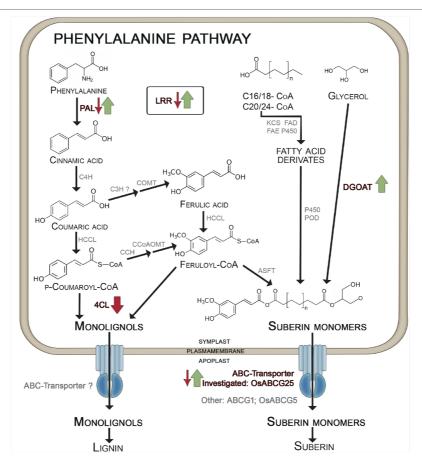


FIGURE 6 | Differential expression of genes involved in exodermal CB development in mutants of OsABCG25. KO mutant: small arrows; OE mutant: big arrows. Phenylalanine-ammonia-lyase (PAL) catalyzes the formation of cinnamic acid which is metabolized by cinnamate-4-hydroxylase (C4H) to coumaric acid. Ferulic acid is built by coumaryl-CoA-3-hydroxylase (C3H) and caffeic acid O-methyltransferase (COMT). Hydroxycinnamate-CoA-ligase (HCCL) catalyzes the formation of coumaryl-CoA and feruloyl-CoA which are converted to monolignols by 4-coumarate ligase (4CL) (Zhong et al., 1998; Eckardt, 2002). From coumaryl-CoA also feruloyl-CoA is generated by p-coumaroyl CoA 3-Hydroxylase (CCH) and caffeoyl-CoA O-methyltransferase (CoAOMT) which is bound to suberin monomers by aliphatic suberin feruloyl transferase (ASFT). The aliphatic components of suberin monomers originate from fatty acid derivates and glycerol, which is incorporated by diacylglycerol O-acyltransferase (DGOT) (Franke et al., 2005; Franke and Schreiber, 2007; Li-Beisson et al., 2013). Both, monolignols and suberin monomers are transported by ABC-transporter from the symplast into the apoplast. We suggested that the investigated OsABCG25 transports suberin monomers. Other suberin transporters are ABCG1 (Landgraf et al., 2014) and OsABCG5 (Shiono et al., 2014). Further proteins are: CCH, p-coumaroyl CoA 3-hydroxylase; FAD, fatty acid desaturase; FAE, fatty acid elongase; KCS, β-ketoacyl-Coa synthase; LRR, leucine-rich repeat family protein; P450, cytochrome P450 monooxygenase; POD, peroxidase. Arrows indicate significant regulation in KO/OE mutants vs. +WT/-WT (p-value > 0.01), green stands for upregulation, red stands for downregulation. The scheme based on Eckardt (2002) and Franke and Schreiber (2007).

transporter *OsABCG5* (LOC\_Os03g17350) shows 72% sequence similarity on a protein level to the ABC transporter in this study. *OsABCG5* is necessary for the CB formation in the exodermis (Shiono et al., 2014), confirming the need of the ABC-transporter for suberin- transport and subsequent CB development.

The LRRs are widely described to be involved in many functions, such as signal transduction, sensing, pathogen-response (Shiu and Bleecker, 2001; Shiu et al., 2004) or even development of root exodermal cells (Huang et al., 2012). It was shown that another LRR, the *Docs1*, belonging to the LRR RLK group in the LRR II subfamily, is necessary for the formation of sclerenchyma and exodermis in young lateral

roots of rice. The LRR in this study belongs to the same structure group and subfamily with an LRR-domain, protein-kinase and transmembrane domain similar to *Docs1*. The LRR was upregulated through Si and by OE of the ABC-transporter. The LRR was downregulated in KO. This indicates a role of LRR in CB development.

# Suberization, Oxidation Power, and Fe Uptake

The suberin analysis in this work showed the typical pattern of rice root aliphatic components (Schreiber et al., 2005a,b).

The OPR contained the typical abundant  $\omega$ -hydroxy acids for rice in C16, 28 and 30. C24 diacids also appeared as expected, but were not different between genotypes (Supplementary Figures S2, S3). However, the total aromatic suberin amount, both coumaric acid and ferulic acid, was lower in KO (Supplementary Figure S4). This may indicate a general disturbance of cell wall metabolism in OPR.

The supply with Si enhanced the development of the CB (Figure 3), as was previously described. It was hypothesized that Si crosslinks phenols with cell walls or induces precipitation of the phenols leading to an enhanced formation of CB (Fleck et al., 2011, 2015). Furthermore, it was shown that Si promotes the deposition of aliphatic and aromatic compounds synchronously in the exodermis as investigated by serial cuttings stained with Berberine-Aniline-Blue and Fluorol Yellow 088 (Fleck et al., 2015). Contrarily, *Arabidopsis thaliana*, a species without CB as an exodermal diffusion barrier, first develops lignin monolayers and later suberin lamellae in the endodermal CB (Naseer et al., 2012). Thus, in the early stages of CB development, CB in the exodermis consists of both suberin and lignin, while it is lignin in the endodermis.

A significant enhanced development of the CB could also be observed in the OE mutant, but no difference compared to the WT occurred in the KO mutant (**Figure 3**). These findings agree well with the fact that the OE of the ABC transporter stimulated the expression of PAL and DGOAT, supplying metabolites for the formation of suberin (**Figure 2**). The CB development in the KO plant was not different from the WT, suggesting that the function of the knocked-out transporter may be substituted by other ABC transporters.

The different CB in Si-supplied WT and in mutants was also reflected in the area of the ROL (Figure 4). The enhanced CB development in Si-supplied WT plants and in the OE mutant resulted in a clearly decreased ROL. The WT plants grown in -Si solution had an oxidation zone along the whole root length, whereas the oxidation zone in WT/+Si and OE/-Si was limited to the first 5 cm from the root tip and to the zone 7-10 cm, where lateral root development starts. The oxidation zone was unaffected under +Si in the KO mutant; this fits with the observation that CB development was not affected. Oxygentransport in rice is provided by aerenchyma from the upper plant organs to the root tip (Nishiuchi et al., 2012). Exodermal CB functions as a diffusion barrier reducing the ROL (Kotula et al., 2009; Fleck et al., 2011). Differential CB development depending on Si supply and mutant was also paralleled by variations of the Fe concentration in shoot matter, which was reduced in WT plants by Si supply and in the OE mutant, but was unaffected in the KO mutant (Figure 5). This agrees with previous findings that Si supply in the nutrient solution reduces

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the concentration of Fe, Mn and other nutrients by 20-50% in leaf DM (Ma and Takahashi, 1990; Dufey et al., 2013). Si, supplied as monosilic acid, can bind with Cu<sup>2+</sup>, Mn<sup>2+</sup> or Fe<sup>2+</sup> (Stevic et al., 2016). Such binding to monosilic acid may also occur with Fe<sup>EDDHA</sup>, as was supplied in this research, and may decrease the bioavailability in rice, a strategy II species. Iron uptake in strategy II species grown in nutrient solution is assumed to take place through binding of Fe<sup>3+</sup> to deoxymugineic acid in the apoplast of roots (Takagi, 1976; Bienfait et al., 1985) and subsequent uptake by yellow stripe-like transporters (Koike et al., 2004; Inoue et al., 2009; Lee et al., 2009). The flux of Fe from the nutrient solution into the apoplast may be impaired by the Si-enhanced development of the exodermis. This conclusion is supported by the observation in the OE mutant that the increased development of exodermal CB resulted in a decreased Fe concentration in shoot matter (Figure 6), which is in line with the consideration of the exodermal CB as a diffusion barrier controlling the ion flow into the apoplast (Faiyue et al., 2010). Exodermal CB hampers the apoplastic pathway of water and Na uptake (Yeo et al., 1987; Steudle, 2000) and it was shown that Si supply reduces the apoplastic Na transport across the root of rice (Yeo et al., 1999; Gong et al., 2006; Krishnamurthy et al., 2009). The presented research confirms the function of OsABCG25 and indicates the involvement of genes related to the phenylpropanoid pathway, such as PAL, DGOAT and 4CL in the Si-promoted formation of CB. The OE of the ABC transporter as well as silicic acid supply enhanced the CB formation in the exodermis leading to a decrease of both, ROL and Fe uptake. This supports the view that the exodermis acts as diffusion barrier controlling fluxes in and out of roots.

#### **AUTHOR CONTRIBUTIONS**

MH, MS, and AF designed the research; MH, AF, EB, and NN performed the experiments; LS provided reagents and helpful discussions and MH, MS, and AF wrote the manuscript.

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#### SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2017.00671/full#supplementary-material

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Supplementary Material

# An ABC Transporter is Involved in the Silicon-Induced Formation of Casparian Bands in the Exodermis of Rice

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#### **Supplementary Data**

Supplementary Tab. 1: Left and right primer of the wildtype primer pair (W-primer) for all mutant lines

| Mutant line | Sequence of left primer | Sequence of right primer |
|-------------|-------------------------|--------------------------|
| 1B-04415    | ATGACGTGGTGGCTGACATA    | GTCGGACACTCCGGAGATAA     |
| 3A-14487    | TAGTGCTCCACTGGTTGCTG    | CCCACTGATCTCTCTCTCGC     |
| 2D-41110    | TGTGGTTCAATTAAACGGCC    | AGGAGTACTCGCAATCCAGC     |
| M0060856    | GCGCCTACTGACCTACGTATG   | CTCGCACTCCATTTCAGTACC    |
| 4A-50856    | TCAAGAAACCCTCGACCATC    | TCGTACCAGCTAGGACTCAAAG   |
| 5A-00450    | TCAATTCGATCCGTTAATTCG   | TGCCCTCAACAACACACAAT     |
| 5A-00464    | AATTTAAGGGCGCCATGCTG    | CACATTTGGTGGTGTTCGGA     |
| 3A-01911    | CCCAAATGCAAGAAACGATC    | CTTCGCTTTGAAGGTCTTGC     |
| M0038578    | GAGCCTTGAATTTGGCAATC    | CAAATCCCTTGGGTTGTC       |
| 3D-01082    | CACCGCGAGTTCATTTCTTT    | TGAGGATGGGTTACCGAAAA     |

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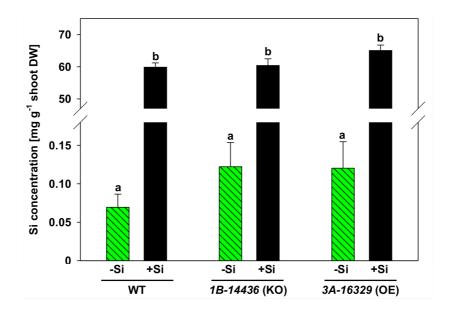
| 3A-01215 | CGAATGTTCAGAGACGGTGA   | TATAGATGCGTGTCGCTTGC   |
|----------|------------------------|------------------------|
| 3A-02897 | ACGTGGCATCATTTTGACTG   | TCAATTCACTCAAACTGCGC   |
| 3A-08589 | TGCTAGTCACCGTTCACCAC   | TTTTATCCGCATTGTACGGG   |
| 3A-06124 | ATTTTTGGCATTCCTGCAAC   | ATCAATCTGAACAGCCCCTG   |
| 3A-16329 | CAGAGGCCAAGGAGTTGAAC   | TGATGCCTGCAAGTTTCATC   |
| 3A-16331 | CTGGGATCCAATCCATTCAG   | GAGTAAGTTGCCACTCCCAAAC |
| 3A-02127 | GGCTAACGCAGACAACTGTG   | CTCCGCATGGAGAAGAAGAC   |
| 3A-60593 | CTCACCGGGAAGACGAGTAA   | CAATTTGCAGCAACTGGATG   |
| 2D-00893 | AGCGAAAGAGGGAAGTTGGT   | CGAAAGGAAGGAGAAGGAG    |
| M0033740 | ATGCGCGACGCGAACGACTT   | GCACTGCATGACGCCCTCGT   |
| M0058854 | AACCGGAATCGGATCCACCGAA | ACAGCTTCATGGTCCGGCTAGT |
| 2A-20141 | GGGTGCTAGATTGAAATATGCC | AAAGGAAGGGTGATGACGTG   |
| M0066685 | TCGCGTAAATACGCAACATC   | GGGAGGAGAGTTGGGAACTC   |
| 1B-14436 | GGACAGTGCTTCCTCTCAGC   | GGCGTTGCTTAGTTACCCTG   |

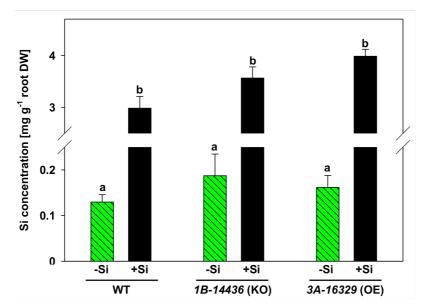
## Supplementary Tab. 2: Insert specific primers (I-primer)

| Suitable for mutants from | Sequence                 |
|---------------------------|--------------------------|
| Postech                   | AACGCTGATCAATTCCAC       |
| Postech                   | CGCGGCGTGCTTTGGAAATGT    |
| Postech                   | ACGAAATGGCCTCCTTGCGGT    |
| TRIM                      | CGAAAAGTTCGACAGCGTCT     |
| TRIM                      | ACATTGTTGGAGCCGAAATC     |
| TRIM                      | CGAAAACGGCAAGAAAAGCAGTC  |
| TRIM                      | AACGGTTCAGGCACAGCACATCAA |

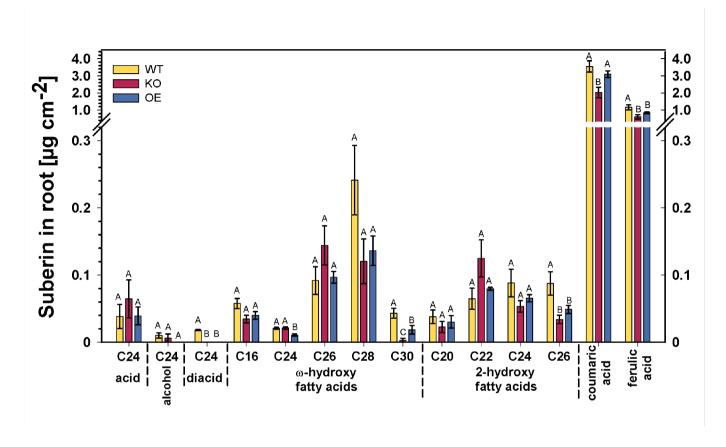
## **Supplementary Tab. 3:** Primer for qRT-PCR

| Gene  | Identifier     | Forward Primer [5'-3']   | Reverse Primer [5'-3']  |
|---|----------------|--------------------------|-------------------------|
| 4-coumarateCoA<br>ligase-like 6 (4CI)           | LOC_Os01g67540 | tgaggcaaccgggtgcatacctta | aagccacacggcgcactttctt  |
| Phenylalanine<br>ammonia-lyase (PAL)            | LOC_Os02g41680 | tcacaagctcaagcaccatc     | ctcaccaagcttcttggcat    |
| Ef1alpha  | LOC_Os03g08010 | tcaagtttgctgagctggtg     | aaaacgaccaagaggagggt    |
| Glycerol-3-phosphate acyltransferase (AT)       | LOC_Os05g20100 | tgctgaacaagcccatcactgc   | tggcgcaatcaactccgatacc  |
| Diacylglycerol O-<br>acyltransferase<br>(DGOAT) | LOC_Os06g22080 | acatgttcgccatgaaccaggca  | tgccctgttgggatcgaagcac  |
| Peroxidase precursor (POD)                      | LOC_Os06g16350 | cagcgccatggacagccaca     | acggtgtcggccgtggagta    |
| Peroxidase precursor (POD)                      | LOC_Os08g02110 | tcctgaattgcccgccttagctct | tcacaaagacgcggccacgaaa  |
| ABC-transporter                                 | LOC_Os10g30610 | atcatctaatgaggcacggc     | tcattgtctggctgcagaac    |
| Leucine-rich repeat family protein (LRR)        | LOC_Os11g14050 | atcaggcaccataccaagccagc  | tgggaggaatgccgccagtgaaa |



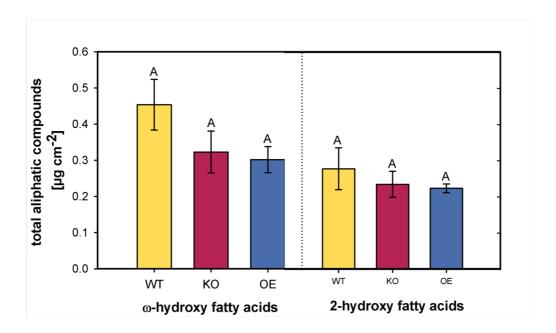


**Supplementary Figure 1:** Silicon concentrations in shoot and root of wildtype (WT) plants and the insertion lines 1B-14436 (KO) and 3A-16329 (OE) grown in nutrient solution with or without Si supply. Different letters indicate a significant difference between Si treatments of a genotype; t-test with p < 0.05.



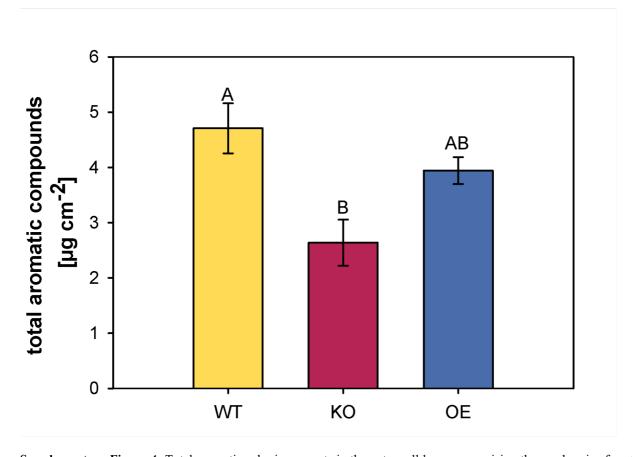
**Supplementary Figure 2:** Suberin amounts in the outer cell layers comprising the exodermis and sclerenchyma of root zone 4-6 cm in wildtype (WT) plants, knockout (KO) mutant, and overexpression (OE) mutant. Amounts were determined via GC-FID. Data are mean  $\pm$  s.e., n = 4. Different letters indicate significant differences between treatments at p < 0.05 using Bonferroni-adjustment.





**Supplementary Figure 3:** Total aliphatic suberin amounts in the outer cell layers comprising the exodermis and sclerenchyma of root zone 4-6 cm in wildtype (WT) plants, knockout (KO) mutant, and overexpression (OE) mutant. Amounts were determined via GC-FID. Data are mean  $\pm$  s.e., n = 4. Different letters indicate significant differences between treatments at p < 0.05 using Bonferroni-adjustment.

## Supplementary Material



Supplementary Figure 4: Total aromatic suberin amounts in the outer cell layers comprising the exodermis of root zone 4-6 cm in wildtype (WT) plants, knockout (KO) mutant, and overexpression (OE) mutant. Amounts were determined via GC-FID. Data are mean  $\pm$  s.e., n = 4. Different letters indicate significant differences between treatments at p < 0.05 using Bonferroni.

# **Chapter III**

## **Silicon Reduces The Iron Uptake in Rice**

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#### **Contributor Roles:**

<u>Martin Hinrichs</u>: Conceived and designed the experiments; performed the experiments; analyzed the data; contributed reagents/materials/analysis tools; wrote the manuscript.

Ngoc S. Ngo: Performed the experiments

Manfred K. Schenk: Conceived and designed the experiments; wrote the manuscript.

## Silicon reduces the iron uptake in rice

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#### 1 Abstract

Gramineous plants take up silicon (Si) that enhances the formation of exodermal Casparian bands (CBs) in the roots of rice (*Oryza sativa* L.). Furthermore, it is known that Si supply reduces the concentration of Fe in rice shoots. We hypothesized that the Si-enhanced CB formation in the exodermis reduces in the flux of Fe in the apoplast and the uptake of Fe loaded deoxymugineic acid. Thus, the effect of silicic acid supply at varied Fe concentrations and Fe forms was investigated in nutrient solution. The Fe concentrations in the shoot and apoplastic Fe concentrations in the root were determined and an Affymetrix GeneChip experiment was carried out together with semiquantitative real time PCR measurements for observation of plant transcriptomic reactions. Additionally, the Fe uptake of an overexpression mutant of Os*ABCG25* with an enhanced exodermal CB formation was investigated.

The application of silicic acid reduced the Fe concentrations in shoot DM independently of the supplied Fe concentration and Fe form. As a reaction to the Fe shortage, the full cascade of Fehomeostasis-related genes in the roots was upregulated. Silicic acid supply also decreased the apoplastic Fe concentrations in roots. In addition, an overexpression mutant of Os*ABCG25* with an enhanced CB formation showed a reduced uptake of Fe in excess Fe conditions. The results suggest that the Si-induced CB formation in the exodermis hampers the flux of Fe into the apoplast of the cortex and, thus, Fe uptake of rice grown in nutrient solution which is reflected in the upregulation of Fe homeostasis-related genes.

Keywords: apoplast, apoplastic bypass flow, Casparian bands, exodermis, iron homeostasis, rice, silicon in agriculture, strategy II,

#### 2 Introduction

Silicon (Si) is the second most abundant element in the soil crust and many plants can take up and accumulate Si (Epstein, 1999) which has beneficial effects, such as a higher resistance to herbivores or powdery mildew, reduced transpiration rate, a higher drought resistance and a higher stability of the leaves (Ma et al., 2001; Ma and Yamaji, 2008). Another effect of Si is the earlier formation of the exodermal Casparian bands (CBs) which appear in submerged grown rice (Vaculik et al., 2009; Fleck et al., 2011). The CBs are depositions of suberin and lignin in anticlinal cell walls of the endodermis of all higher plants and of the exodermis of many plants (Ma and Peterson, 2003; Schreiber et al., 2005). Endodermal CBs control the unselective bypass flow of water and ions into the vascular bundle (Zimmermann and Steudle, 1998). Exodermal CBs are described in rice as controlling radial oxygen loss and water flux (Armstrong, 1971; Steudle, 2000). It is also indicated in the literature that the exodermal CBs could affect in nutrient acquisition. Due to the late occurrence of CBs during root development and the disruptions of the exodermis from lateral roots, it is mentioned that the effect of exodermal CBs in ion exchange should not be overestimated (Enstone and Peterson, 1997; Sattelmacher, 2001). Accordingly, the flux of apoplastic tracer, such as trisodium 3-hydroxy-5,8,10-pyrene trisulfonat or cis-absicid acid was not hampered by the exodermal CBs (Zimmermann and Steudle, 1998; Freundl et al., 2000). But, the exodermal CBs are also described as a diffusion barrier reducing the flux of ions into the cortex (Gierth et.al., 1999; Yeo et al., 1999; Gong et al., 2006; Faiyue et al., 2010) and to have a function in controlled substance exchange (Stasovski and Peterson, 1991; Kamula et al., 1994; Garcia et al., 1997), while Ma and Peterson (2003) notice the positive effects of the exodermal CBs for water management. Furthermore, it has been shown that Si supply reduces the bypass flow of sodium and, thus, the sodium uptake declines (Yeo et al., 1999; Ma et al., 2001; Gong et al., 2006)

Rice has exodermal CBs which have a specific function regarding the aerenchyma, which is a root tissue developing from collapsing cortical cells to ensure the oxygen supply of the root (Nishiuchi et al., 2012; Leite et al., 2017). Exodermal CBs block the radial oxygen loss in adventitious roots (Fleck et al., 2011). Previously it was shown that Si enhances the formation of exodermal CBs in adventitious roots of rice and clearly reduces the radial oxygen loss (Fleck et al., 2011; Hinrichs et al., 2017). The exodermal CB formation starts with/ without Si application 4-5/8-10 cm from the root tip in the anticlinal cell walls and is completed at 7-8/12-13 cm distance from the root tip, respectively (Fleck et al., 2011, 2015).

Silicon also affects nutrient uptake in rice. Ma and Takahashi (1990) reported that Si supply reduced the Fe uptake and Dufey et al. (2013) described less Fe uptake under Fe excess conditions but causal relationships are still not clear. Rice, a gramineous plant, follows strategy II in Fe uptake. In strategy II, plants secrete phytosiderophores, such as deoxymugineic acid (DMA), into the rhizosphere that bind to hardly available Fe<sup>III+</sup>. This complex is taken up by yellow stripe-like (YSL) transporters at the root epidermis and, most probably, in the root cortex (Koike et al., 2004; Ogo et al., 2014b; Senoura et al., 2017). The rhizosphere is different in plants grown in nutrient solution to those grown in soil since the whole pot volume can be defined as rhizosphere, because of the high diffusion coefficient in water. Thus, DMA is extremely diluted and less effective for Fe acquisition. In nutrient solution, the binding to DMA and the subsequent uptake is discussed to take place in the apoplastic space of the cortex (Zhang et al., 1991).

The aim of this work is to identify the reasons for the lower Fe content in the leaves of plants grown with Si. We hypothesize that the earlier CB formation in the exodermis reduces the amount of Fe in the apoplast and, thus, reduces the uptake of DMA-loaded Fe.

#### 3 Material and methods

#### 3.1 Plant material, growth conditions and harvest

Seeds of Oryza sativa ssp. Japonica cv. Selenio were first surface sterilized in 70 % ethanol for 1 min and then for 30 min in 3.5 % NaClO, followed by washing in sterile water three times. Germination was carried out for several days between two layers of filter paper standing in sterile tap water for 14 d. Seedlings were transferred to nutrient solution in 5-L pots containing  $0.43 \text{ mM NH}_4\text{NO}_3$ ,  $0.32 \text{ mM NaH}_2\text{PO}_4 \times 2 \times \text{H}_2\text{O}$ ,  $0.51 \text{ mM K}_2\text{SO}_4$ ,  $1 \text{ mM Ca}(\text{NO}_3)^2 \times 4 \times \text{H}_2\text{O}$ ,  $1.6 \text{ mM MgSO}_4 \times 7 \times \text{H}_2\text{O}, \ 1.82 \text{ } \mu\text{M MnSO}_4 \times \text{H}_2\text{O}, \ 0.03 \text{ } \mu\text{M (NH}_4)_6\text{Mo}_7\text{O}_{24} \times 4 \times \text{H}_2\text{O}, \ 9 \text{ } \mu\text{M}$  $H_3BO_3$ , 0.6  $\mu$ M ZnSO<sub>4</sub>× 7× $H_2O$  and 0.15  $\mu$ M CuSO<sub>4</sub>× 5× $H_2O$ . Iron was added as Sequestren<sup>TM</sup> (Fe<sup>EDDHA</sup>) for Fe gradient experiment in three different levels, low Fe (3.58 µM Fe<sup>EDDHA</sup>), optimal Fe (35.81 µM Fe<sup>EDDHA</sup>) and high Fe (179.05 µM Fe<sup>EDDHA</sup>) corresponding to 0.2, 2 and 10 mg Fe, respectively, in nutrient solution. An amount of 2 mg Fe L<sup>-1</sup> was added as Sequestren<sup>TM</sup> (Fe<sup>EDDHA</sup>), Fetrilon<sup>TM</sup> (Fe<sup>EDTA</sup>) and iron sulfate (Fe<sup>II</sup>SO<sub>4</sub> × 7×H<sub>2</sub>O) for the Fe form experiments. Plants were cultivated for three weeks as in the Fe gradient experiment, and the last week with 4.475 mM Fe supplied as  $Fe^{II}SO_4 \times 7 \times H_2O$  for the growth of the mutants of OsABCG25. Please see Hinrichs et al. (2017) for characterization of OsABCG25. The Si was applied as silica gel and the concentrations were 3 or 30 mg L<sup>-1</sup>, resulting in Si concentrations of 0.1 and 60 mg g<sup>-1</sup> shoot DM, respectively (data not shown). It was not possible to use less than 3 mg L<sup>-1</sup> Si for technical reasons. The pH value of the nutrient solution was adjusted to 6.0 by the addition of 10 % H<sub>2</sub>SO<sub>4</sub> or 5 M KOH. The nutrient solution was renewed weekly for the first two weeks, after two weeks, and subsequently the nutrient solution was changed twice a

week until harvest. Plants were cultivated in a climate chamber (photoperiod 16/8 h light/dark; temperature,  $25/20^{\circ}$ C day/night; 75 % relative humidity and a light intensity of 220  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup>) for 28 d in nutrient solution.

After 28 d in nutrient solution, the root zones 4-6 cm from the the root tip were harvested, transferred immediately to liquid nitrogen and stored at -80°C for transcript analysis. Only adventitious roots were harvested for Fe determination in the apoplast. Shoot and root were separated, dried at 60°C for 4 d and ground for element analysis. The content of chlorophyll was measured spectrometrically using an N-Tester (Yara, Dülmen, Germany).

#### 3.2 Fe determination apoplast

Adventitious roots were separated and Fe on the root surface was removed through rinsing in anaerobe washing solution (12 mM  $Na_2S_2O_4$ , 10 mM MES, 0.5 mM  $Ca(NO_3)_2$ , pH = 5.5) for 5 s. Subsequently, the roots were washed three times in  $H_2O$  and transferred into 21 ml anaerobe reaction solution (1.5 mM 2.2-Bipyridyl, 10 mM MES, 0.5 mM  $Ca(NO_3)_2$ , pH = 5.5), as described in Bienfait et al. (1985). The reaction solution with the samples was deoxygenized by  $N_2$  bubbling in an Erlenmeyer flask covered with a cotton plug for 2 min. After 2 min, 1 ml of a cooled 250 mM  $Na_2S_2O_4$ -solution was added using a syringe and incubated by gently shaking for 5 min at room temperature. After incubation, the roots were removed, and the reaction was stopped. The washing solution and reaction solution were stored on ice with constant  $N_2$ -bubbling until usage. The Fe was determined by measuring the A520 of the filtrated reaction solution.

#### 3.3 Transcript analysis

Frozen root material was ground under liquid nitrogen and the RNA was isolated using NucleoSpin RNA Plant (Macherey-Nagel, Düren, Germany), following the manufacturer's instructions. The RNA quality was determined electrophoretically by 1 % Agarosegel and fluoretically using a Nanophotometer (Implen, Munich, Germany). The RNA (1  $\mu$ g), oligo(dT)18-Primer (0.25  $\mu$ g) and random hexamer primer (0.25  $\mu$ g) were used to synthesize first-strand cDNA using the Revert Aid<sup>TM</sup> H Minus First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany), following the manufacturer's instructions for GC-rich templates.

The RNA was additionally measured by capillary electrophometric by a bioanalyzer (Agilent 2100 bioanalyzer, Agilent Technologies) using a RNA 6000 nano assay in the GeneChip experiment. Only samples with a RNA integrity number over 9.5 were used for the GeneChip experiment. A set of sixteen GeneChips were used for the experiment, consisting of two treatments (+Si/-Si), four biological repeats and two technical repeats of the Affymetrix GeneChip experiment. One biological repeat consisted of a pool of the RNA of five plants grown in the same 5-L pot.

An amount of 50 ng cDNA was used as a template in 10 µl reaction mix of the SYBR Premix Ex Taq (Tli RNase H Plus) (Takara Bio Europe SAS, Saint-Germainen-Laye, France) and 0.2 µM forward and reverse primer in the quantitative real time PCR (qPCR) experiments. The qPCR runs were performed in the CFX96 cycler (Bio-Rad, München, Germany), using an initial 95°C step for 30 s, followed by 40 cycles of 95°C for 5 s, and 60°C for 30 s, and a final melting curve procedure with a stepwise increment of 0.5°C ranging from 65 to 95°C.

The eukaryotic elongation factor 1-alpha (eF1- $\alpha$ , LOC\_Os03g08020.1) and Ubiquitin-40S ribosomal protein S27a-1 (UBQ5, LOC\_Os01g22490) were used as an endogenous control due to its stable transcript abundance in rice (Jain et al., 2006; Jain 2009). The geometric mean of the Ct values of the two endogenous control genes was calculated according to Vandesompele et al. (2002), and this was used to calculate the fold-over reference of the different samples (2<sup>-delta Ct</sup>). A list of primer sequences used can be found in Supplementary Table S1. The primer was tested for specificity in a ten times dilution series starting with 50ng cDNA and then sequencing of the PCR product. Three technical and four biological replicates were used for each target in qPCR. The relative quantity was calculated using the R-Macro "qpcrmix" (Steibel et al., 2009), based on the  $2^{-\Delta\Delta CT}$  method.

#### 3.4 Chemical analysis

For the determination of the Si concentrations in the shoot and root, 200 mg dried plant matter was digested in 3 ml 65 % HNO<sub>3</sub>, 2 ml H2O and 2 ml 30 % H<sub>2</sub>O<sub>2</sub> in a microwave for 12 min at 190°C, then diluted with 20 ml 10 % NaOH, neutralized with HNO<sub>3</sub> (Haysom and Ostatek-Boczynski, 2006) and filled up to a final volume of 100 ml.

In order to determine the Fe, 50 mg of dried and ground shoot matter was digested in 2 ml 65 % HNO<sub>3</sub>,  $2 \text{ ml } H_2O$  and  $0.5 \text{ ml } 30 \% H_2O_2$  in a microwave for 25 min at  $190^{\circ}\text{C}$  and then diluted with distilled water to 25 ml.

The Si and Fe concentrations in the plant extracts and nutrient solutions- were measured by ICP-MS (7500c Agilent Technologies).

#### 3.5 Statistical analysis

All treatments were replicated four times unless stated otherwise and the mean of the treatments were compared using t-test, Tukey or Bonferroni test utilizing Sigma Plot (Systat Software Inc., San Jose, USA). The statistical qPCR analysis was performed using the R-Macro of Steibel et al. (2009). A students t-test was used with unpaired correlated p-Value for the raw Affymetrix GeneChip data using "GeneSpring" Software (Version 12.0, Agilent). A ranking of candidates was carried out using the "Omics Explorer" (Version 3, Qulcore).

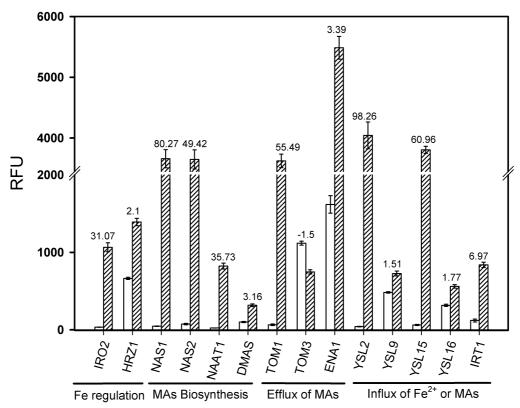
The Affymetrix experiment, including the RNA quality control, GeneChip hybridization, normalization with GeneSpring Software and ranking with the Omics Explorer, was carried out by Dr. Dittrich-Breiholz at the Research Core Unit "Transcriptomics," Institute of Cell Biochemistry, Hannover Medical School.

#### 4 Results

## 4.1 Silicic acid induced differential gene expression

Rice plants were grown with high (+Si, 30 mg L<sup>-1</sup>) or low Si (-Si, 3 mg L<sup>-1</sup>) in nutrient solution for 28 d. The zone 2-6 cm from the tip of adventitious roots was harvested, since here CB development in the exodermis is clearly enhanced by Si supply, and an Affymetrix GeneChip was used to identify differentially regulated genes. We observed 172 upregulated and 207 downregulated genes due to Si treatment with a minimal fold change of 2 at a p-value cut-off of 0.01 (Supplementary Fig. S1). Fourteen of the upregulated genes were related to the Fe homeostasis in rice (Fig. 1): The Fe-regulating transcription factors Os*IRO2* (LOC Os01g72370.1) and Os*HRZ1* (LOC Os01g49470.2). Additionally, genes for the

desoxymugineic acid (DMA) biosynthesis, such as nicotianamine synthase 1/2 (OsNASI, LOC Os03g19427.1; OsNAS2 LOC Os03g19420.2), nicotianamine aminotransferase 1 LOC Os02g20360.1) and deoxymugineic (OsNAAT1,acid synthase LOC Os03g13390.2) were upregulated. The mugineic acid (MA) efflux transporter OsTOM1 (LOC Os11g04020.1) was similarly upregulated, while the expression level of OsTOM3 was decreased. The nicotianamine efflux transporter OsENA1 responsible for Fe-MA mobilization in the vacuole was also upregulated. An increased expression level was furthermore observed for genes related to the influx of Fe-MA, such as OsYSL2 (LOC Os02g43370.2), OsYSL9 (LOC Os04g45860.1), OsYSL15 (LOC Os02g43410.2) and OsYSL16 (LOC Os04g45900.1). The  $Fe^{2+}$  transporter 1 (OsIRT1) was also upregulated. A differential regulation of genes involved in Fe-MA storage and sequestration was not observed (OsVit1.1/1.2, LOC Os04g38940.1/LOC Os09g23300.1; OsFer1/2, LOC Os11g01530.1/LOC Os12g01530.1).

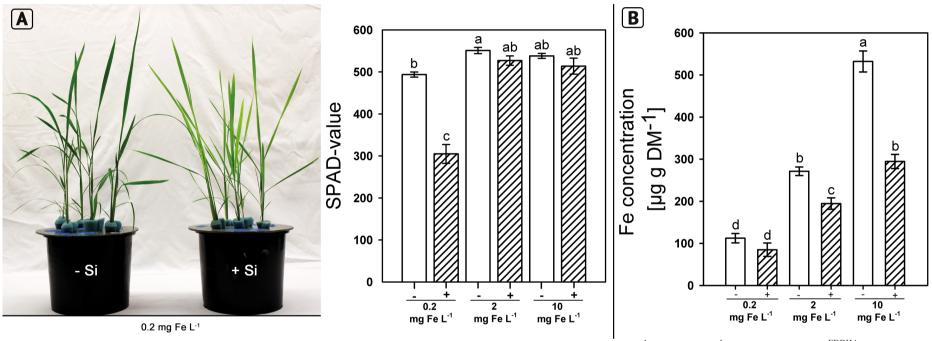


**Figure 1:** Regulation of Fe homeostasis-related genes through silicic acid (Si) in rice roots from the Affymetrix GeneChip experiment. Ruled bars represent the Si-treated plants grown with Si (30 mg L<sup>-1</sup>) in nutrient solution; white bars represent plants grown with low Si concentrations (3 mg L<sup>-1</sup>). The relative fluorescence unit (RFU) is shown; bars indicate the standard error (SE). Numbers above the bars represent the foldchange (FC). Abbreviations of the genes are: DMAS: Deoxymugineic acid synthase; ENA1: Efflux of nicotianamine 1; HRZ1: Haemerythrin motif-containing RING-& Zinc finger protein 1; IRO2: Iron-related transcription factor 2; IRT1: Iron related transporter 1; NAAT1: Nicotianamine aminotransferase 1; NAS1: Nicotianamine synthase 1; NAS2: Nicotianamine synthase 2; TOM1: Transporter of mugineic acid 1; TOM3: Transporter of mugineic acid 3; and YSL2/9/15/16: yellow stripe like (YSL) transporter 2/9/15/16.

#### 4.2 Fe nutrition as affected by silicic acid at varied Fe supply

The observations suggested that Si supply induced a Fe shortage in rice plants. Thus, we investigated the Fe uptake and the gene expression as affected by Si nutrition at different Fe levels and Fe forms. Plants grown for 28 d in +Si at the lowest Fe supply showed Fe deficiency symptoms (Fig. 2A) compared to the other treatments, which was also reflected in the SPAD

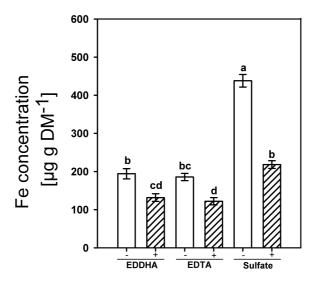
values (Fig. 2A). Furthermore, the Fe concentration in shoot DM was reduced by Si supply in all Fe levels and reduction varied between 25 and 32.4 % at low and high Fe levels, respectively (Fig. 2B).



**Figure 2:** Iron nutritional status of rice plants grown in nutrient solution as affected by silicic acid supply (+Si: 30 mg L<sup>-1</sup>, -Si: 3 mg L<sup>-1</sup>) and Fe supply (Fe<sup>EDDHA</sup>) (**A**) phenotypic differences between rice plants grown in low Fe conditions (0.2 mg L<sup>-1</sup>) and chlorophyll concentration of leaves shown as SPAD value of plants grown with low Fe (0.2 mg L<sup>-1</sup>), optimal Fe (2 mg L<sup>-1</sup>) and high Fe (10 mg L<sup>-1</sup>). (**B**) Iron concentrations in the shoot dry matter (DM). Ruled bars represent the +Si plants; white bars represent plants grown with -Si. Bars indicate the SE. Different letters indicate significant differences between treatments; Bonferroni test with p < 0.05.

Silicic acid supply also reduced the Fe concentrations in shoot DM independently of the Fe form, as can be seen in Figure 3. The Si effect was much stronger for plants grown with Fe<sup>(II)</sup>SO<sub>4</sub> than with Fe<sup>EDDHA</sup> and Fe<sup>EDTA</sup>. The Fe supply in the form of Fe<sup>(II)</sup>SO<sub>4</sub> enhanced the Fe concentration in shoot DM by a factor of two compared to the Fe chelates.

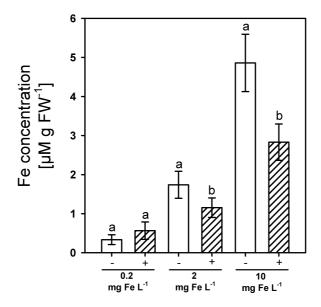
There was no difference in the chlorophyll concentrations (SPAD) at harvest between treatments (Supplementary Fig. 2), but plantlets grown in +Si with Fe<sup>EDTA</sup> or Fe<sup>(II)</sup>SO<sub>4</sub> showed chlorotic leaves at the beginning of the experiment. This effect was not measurable with the SPAD meter due to the small leaf area of the plantlets.



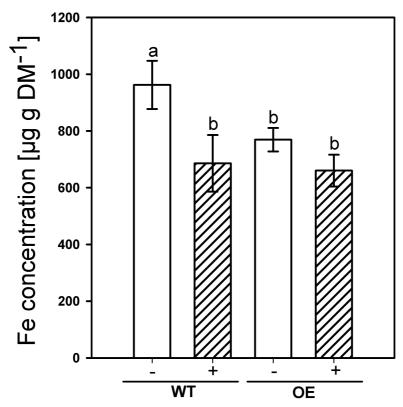
**Figure 3:** The Fe concentration in the shoot DM of rice as affected by the offered Fe form (2 mg  $L^{-1}$ ) and silicic acid supply (+Si: 30 mg  $L^{-1}$ , -Si: 3 mg  $L^{-1}$ ) in nutrient solution. Ruled bars represent the +Si-treatments; blue bars represent -Si treatments. Bars indicate the SE. Different letters indicate significant differences between treatments; Bonferroni test with p < 0.05.

Since silicic acid supply reduced the Fe concentration in shoot DM regardless of the level and the form of Fe supply, we assumed a common mechanism for this Si effect. We hypothesized that the Si-enhanced CB development in the exodermis (Fleck *et al.* 2011, Hinrichs *et al.* 2017) reduced the Fe flux into the root apoplast where Fe is thought to be bound by DMA and then taken up by YSL transporters. Therefore, the apoplastic Fe concentrations were determined (Fig. 4). They clearly increased with increasing Fe supply and were reduced by Si supply at the two higher Fe supply levels.

Additionally, an overexpression mutant of the gene Os*ABCG25* (LOC\_Os10g30610.1) having an enhanced CB development in the exodermis was used and grown under excessive Fe supply to reinforce the hypothesis (Hinrichs *et al.*, 2017). The overexpression mutant grown without Si contained clearly less Fe in shoot DM compared to WT (Fig. 5). The application of Si to WT plants affected the Fe concentration similarly, but the effect was only slightly visible in the mutant.



**Figure 4:** The Fe concentration in the apoplast of adventitious rice roots as affected by Fe supply (Fe<sup>EDDHA</sup>) and silicic acid supply (+Si: 30 mg L<sup>-1</sup>, -Si: 3 mg L<sup>-1</sup>). Ruled bars represent +Si plants; white bars represent -Si plants. Bars indicate the SE. Different letters indicate significant differences between respective treatments; Wilcoxon rank sum test with p < 0.05.

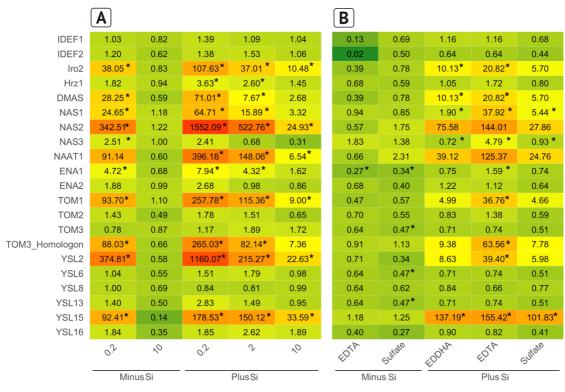


**Figure 5:** The Fe concentration in the shoot of WT and the overexpression mutant of OsABCG25 grown with excessive amounts of Fe (250 mg  $L^{-1}$  FeSO<sub>4</sub>) in nutrient solution. Ruled bars represent the Si-treated plants (+Si, 30 mg  $L^{-1}$ ); blue bars represent plants grown with low Si (-Si, 3 mg  $L^{-1}$ ) concentration. Bars indicate the SE. Different letters indicate significant differences between WT plants grown without Si (-WT) and other treatments; students t-test with p < 0.05.

## 4.3 Gene expression as affected by silicic acid at varied Fe supply

Silicic acid supply hampered the Fe nutrition of rice and this was reflected in the expression of Fe homeostasis genes. About half of the genes investigated were upregulated at the lowest Fe level without Si supply (Fig. 6A), but not differentially expressed at the highest Fe level compared to the optimal level of 2 mg L<sup>-1</sup>. The expression of the same genes which were upregulated in the treatment 0.2 mg Fe L<sup>-1</sup> were also enhanced in all Fe levels by Si supply and the expression declined with increasing Fe concentration in the nutrient solution: The transcription factors OsIRO2 and OsHRZ1, the chelate synthesis genes OsNAS1, OsNAS2, OsNAAT1 and OsDMAS, the DMA efflux transporter genes OsTOM1 and the OsTOM3 homologon (Os12g0132500), and finally the Fe-MA uptake transporters YSL2 and YSL15. The expression of OsENA1 coding for a Fe-DMA/nicotianamine (NA) transporter from the vacuole was also enhanced.

A similar pattern of Si-induced gene expression was observed in the experiment with different Fe forms (Fig. 6B).



**Figure 6:** Regulation of Fe homeostasis-related genes in rice roots as affected by Fe concentration and Fe form grown without Si and with Si (3 and 30 mg Si L<sup>-1</sup>, respectively) in nutrient solution. The  $2^{-\Delta ACt}$  values against the control are shown. A) Gene expression in roots of rice plants grown with Fe<sup>EDDHA</sup> in various concentrations (control = -Si, 2mg L<sup>-1</sup> Fe). B) Gene expression in roots of rice plants grown with 2 mg Fe in the form of Fe<sup>EDDHA</sup>, Fe<sup>EDTA</sup> and Fe<sup>(II)</sup>SO<sub>4</sub> (control = -Si, Fe<sup>EDDHA</sup>). A star indicates significant differences against the control. Green color indicates downregulation and a red colour upregulation compared to the WT grown without Si at optimal Fe conditions (2 mg L<sup>-1</sup> Fe<sup>EDDHA</sup>).

## 5 Discussion

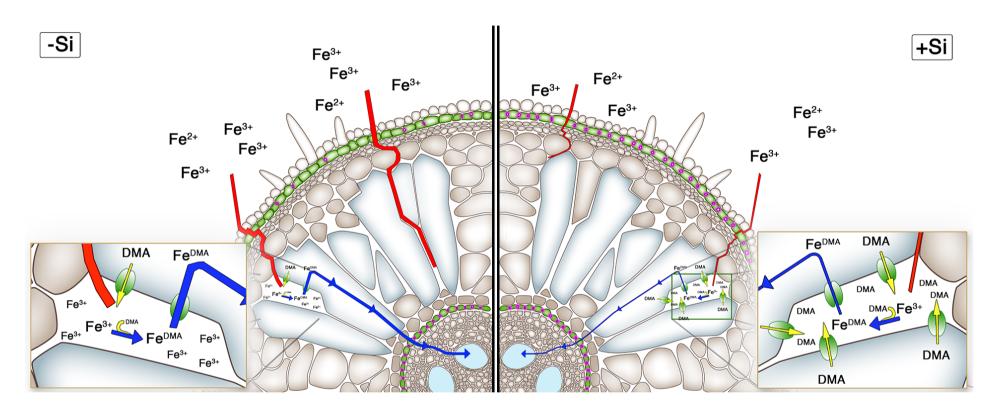
## 5.1 Silicic acid and iron acquisition

Silicon reduced the concentration of Fe in the shoot of plants independently of the supplied Fe concentration and Fe form and triggered Fe deficiency symptoms (Fig. 2+3). This agrees well with previous reports that Si supply decreases the Fe concentration in shoots of rice grown in nutrient solution (Ma and Takahashi, 1990; Dufey *et al.*, 2013). We also observed a reduction of the Fe concentration in the root apoplast (Fig. 4). Thus, we suggest that the Si-enhanced formation of the exodermal CBs led to a decrease of the Fe concentration in the apoplast and reduced Fe uptake.

The formation of exodermal CBs is promoted by Si and CBs occur earlier in the adventitious roots of rice (Fleck et al., 2011) and in the roots of Zea mays, Allium cepa, Tradescantia virginiana and Guizotia abyssinica (Fleck et al., 2015). The endodermal CBs are well described to be an effective barrier for unselective bypass flow of substances from the apoplastic space into the vascular bundle (Stasovski and Peterson, 1991; Yadav et al., 1996; Enstone and Peterson, 1997; Ranathunge et al., 2003) and the exodermal CBs were shown to hamper the flux of oxygen from the aerenchyma into the rhizosphere of rice roots (Kolattukudy and Agrawal, 1974; Colmer and Bloom, 1998; Schreiber et al., 2005; Vaculík et al., 2009; Fleck et al., 2011). Furthermore, it was observed that Si reduces sodium uptake in rice due to a reduced transpirational bypass flow (Yeo et al., 1999; Gong et al., 2006). However, the impact of exodermal CBs in nutrient and water uptake is controversially discussed in the literature. Apoplastic tracers are shown to be permeable in roots with an exodermis, such as trisodium 3hydroxy-5,8,10- pyrene trisulfonat or cis- absicid acid (Zimmermann and Steudle, 1998; Freundl et al., 2000). However, it is discussed that the lateral root formation may interrupt the exodermal CBs, resulting in a reduced barrier function of the exodermis (Ma and Peterson, 2003; Ranathunge et al., 2005). Earlier results from Hinrichs et al. (2017) suggested the exodermal CBs as a diffusion barrier for Fe into the apoplastic space. We observed that Si supply reduced the concentration of apoplastic Fe (Fig. 4), supporting the evidence that CBs act as a barrier for the flux of Fe into the root cortex. The apoplastic Fe concentration was in the range of 1 up to 2 µmol g FW<sup>-1</sup> at optimal Fe conditions, as observed by Bienfait et al. (1985). Furthermore, a mutant overexpressing the ABC transporter OsABCG25 resulting in enhanced CB development (Hinrichs et al., 2017) also had a decreased concentration of Fe in the shoot DM (Fig. 5). The presented results support the hypothesis that the exodermal CBs hamper the flux of Fe from the nutrient solution into the apoplast of the root cortex.

Rice is a strategy II plant (Römheld and Marschner, 1986), releasing DMA by transporters into the rhizosphere of plants grown in soil where DMA binds to Fe ions. The Fe-loaded DMA is taken up into the symplast by transporters located in the root epidermis and in the cortex. Regarding plants grown in nutrient solution, however, the whole solution volume can be considered as rhizosphere because of the large diffusion coefficient and the DMA secreted will be extremely diluted and less effective for Fe acquisition. Thus, Zhang *et al.* (1991) suggested that under these conditions, the uptake of Fe most probably takes place from the apoplast where the excreted DMA binds to Fe and then the complex is taken up into the cortex cells.

A scheme displaying the suggested functions of Si in Fe acquisition of plants grown in nutrient solution is presented in Figure 7. The Si-induced CB formation in the exodermis hampers the flow of Fe into the apoplastic space where less Fe can be bound to DMA and taken up into the symplast. In agreement with this model, Si supply induced upregulation of Fe homeostasis genes.



**Figure 7:** The Fe acquisition in rice roots grown in nutrient solution as affected by Si supply.

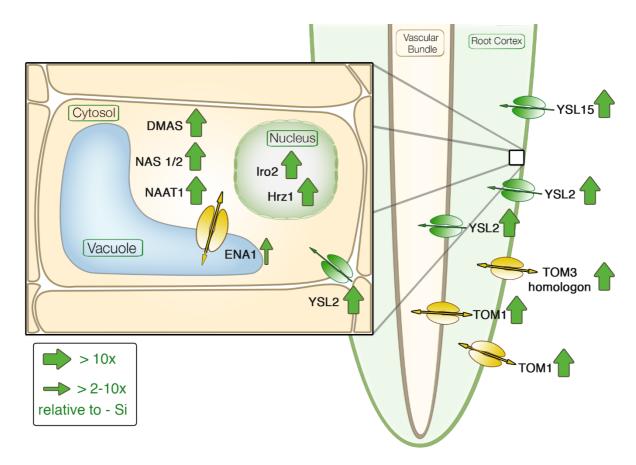
## 5.2 Silicic acid induced differential gene expression

The Si-induced reduction of Fe uptake was also reflected in gene expression. The transcriptomic data displayed the full range of Fe deficiency-induced reaction cascade in rice (Fig. 1 and 6). An overview of the interacting genes and their activity sites is given in Figure 8. The reaction cascade starts with the transcription factors Os*IDEF1* or 2, which both are regulating elements of Os*TOM1*, Os*IRO2*, Os*NAS1* + 2, Os*NAAT1* and Os*DMAS*. The transcript level of Os*IDEF1* + 2 is not influenced by Fe availability, but only the protein binding to the DNA is enhanced during Fe deficiency (Nozoye *et al.*, 2011; Kobayashi and Nishizawa, 2014). This agrees well with our results that Si did not affect their expression levels (Fig. 6). The activity of Os*HRZ1* is enhanced by IDEF1 and is suggested as negative feedback loop (Kobayashi *et al.*, 2013). Putatively, Os*IRO2* activates the biosynthesis of NA and, thus, the synthesis of DMA and MA in subcellular organells through Os*NAS1*, Os*NAS2*, Os*NAAT1* and Os*DMAS* (Bashir and Nishizawa, 2006). The DMA is then transported via OsTOM1 into the apoplast (Nozoye *et al.*, 2011, 2014).

TOM3 was not affected by Si in rice roots, while the TOM3 homologon (Os12g0132500) was highly upregulated, which agrees with Ogo et al., (2014), who found an upregulation of the TOM3 homologon, but not of Os TOM3 in the epidermis of rice roots in Fe-deficient conditions. The putative vacuole transporter OsENA1 (Nozoye et al., 2011) transporting NA was upregulated. Os*ENA1* is thought to mobilize Fe during Fe shortage from the vacuole. Further characterization of OsENA1 is necessary, since the recent research is only based on in vitro motif predictions. Finally, Fe-loaded DMA is taken up by different YSL transporters. Os YSL2 is described as expressed mainly in phloem companion cells for the transport of Fe<sup>II</sup>-NA (Koike et al., 2004). Because of the Si-induced shortage of Fe, OsYSL2 was highly upregulated. Most probably, OsYSL2 is responsible for the uptake and distribution of Fe<sup>II</sup>-NA by Si-induced Fe shortage. No regulation was observed for OsYSL9 and OsYSL13. OsYSL9 was recently shown to be expressed in the cortical cells of roots (Senoura et al., 2017) and OsYSL13 was described in cortex and epidermis cells (Ogo et al., 2014a). Both, OsYSL9 and 13 transporters do not seem to be involved in the Si-dependent reaction. Os YSL15 was highly upregulated, but not Os YSL16; both are located in the exodermis or in the cortex. Os YSL15, transporting Fe<sup>III</sup>DMA, is under Fe deficiency induced in all root parts (Nozove *et al.*, 2014).

## **6 Conclusion**

Silicic acid supply stimulates the formation of exodermal CBs. It is suggested that this barrier reduced the flux of Fe into the apoplastic space where less Fe was available for chelation by DMA and plant uptake. The plant reacted with an upregulation of Fe homeostasis-related genes and a higher production of Fe-chelating substances as a metabolic answer to the Fe shortage. This response was not sufficient to overcome the Si effect, since shoot Fe concentration was still decreased.



**Figure 8:** Schematic figure of Si-induced Fe homeostasis-related genes in rice. Significant data from results given in Figure 5 are shown. A big arrow indicates an upregulation compared to the control (-Si; plants grown without Si) with a minimum of tenfold; a small arrow indicates an upregulation with a minimum of twofold. Please see Figure 1 for abbreviations.

## **Chapter IV**

# Investigations of the Genes Involved in the Si-induced Formation of Casparian Bands in *O. sativa* (L.)

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#### **Contributor Roles:**

<u>Martin Hinrichs</u>: Conceived and designed the experiments; performed the experiments; analyzed the data; contributed reagents/materials/analysis tools; wrote the manuscript.

Björn Feldmann: Performed the experiments

Ewa Schneider: Performed the experiments; contributed reagents/materials/analysis tools

Madita Wandrey: Performed the experiments

<u>Traud Winkelmann:</u> Conceived and designed the experiments; contributed reagents/materials/analysis tools

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## **Chapter IV**

# Investigations of the Genes Involved in the Si-induced Formation of Casparian Bands in *O. sativa* (L.)

## 1 Abstract

Rice is able to accumulate Si, which has several beneficial effects on plant growths. It has been shown previously that Si is able to enhance the formation of the exodermal CB. The genes involved in this Si dependent action are partly unknown. To identify more genes involved in the Si dependent formation of exodermal CB, the same whole genome Affymetrix GeneChip was used as seen in Chapter III. In total, 33 new candidate genes related to the Si dependent formation of exodermal CB were identified. Furthermore, homozygous KO mutants for six ABC transporter, four phenol- and two lipid metabolism-related genes were generated. Additionally, four fully transgenic rice lines carrying a modified CRISPR nuclease (nickase) were generated for the Si dependent exodermal CB formation and a rice transformation protocol was established.

## 2 Introduction

Most higher plants develop CBs between the anticlinal cell walls of the endodermis and the exodermis (Perumalla and Peterson, 1986; Perumalla *et al.*, 1990). While the endodermal CB appears very early after root formation, the exodermal CB development is done from 6-12 cm behind the root tip, if no Si is available (Nishiuchi *et al.*, 2012). Studies showed that Si is able to enhance the formation of exodermal CB, whose formation takes place 2-6 cm behind the root (Fleck *et al.*, 2013). It was possible to identify 19 genes involved in the Si dependent formation of exodermal CB (Chapter II) but the influence of Si on the CB development remains unclear. Thus, an Affymetrix GeneChip analyzes was performed (Chapter III) to identify more genes involved in the Si dependent formation of exodermal CBs. The respective candidate genes were investigated in KO plants.

Oryza sativa (L.) is a sequenced organism (Kawahara et al., 2013) and thus suitable for functional genetic approaches. Several seed banks are available offering seeds of KO mutants. Available mutant seeds have an insertion in the respective gene of interest which results in nonsense transcripts and a KO of the gene due to posttranscriptional modifications. Rice seeds **National** Institute of Agrobiological Sciences, https://tos.nias.affrc.go.jp/index.html.en; Miyao et al., 2003) were propagated from plants carrying a Tos17 retrotransposon. Tos17 integrates itself randomly into the rice genome under tissue culture conditions (Hirochika et al., 1996) and remains stable at its position after integration in normal growth conditions. Rice seeds of the Crop Biotech Institute, Department of Plant **Systems** Biotech, Kyung Hee University, Korea (PFG; http://cbi.khu.ac.kr/RISDDB.html; Jeon et al., 2002; An et al., 2003) carry a random insertion of the T-DNA from a binary plasmid (pGA2715/-17 & pGA2772), which interrupts the coding sequence of a gene in the same manner as *Tos17*. Depending on mutant availability, mutants of both origins were ordered and propagated. In case that a gene of interest was not covered by one of the seed banks, a rice transformation protocol from Aung *et al.* (2013) and Naoko K. Nishizawa (Ishikawa Prefectural University, Tokio, Japan; personal communication) was established. Recently, Brooks *et al.* (2014) showed that CRISPR nucleases are able to induce homozygous modification events in T0 plants. Based on these results a modified CRISPR nuclease (nickase) was used to generate KOs in fully transgenic rice lines.

## 3 Material and methods

## 3.1 Plant material and growth conditions

For the Affymetrix GeneChip experiment, plant culture, harvest, and RNA and cDNA preparation was performed as described in Chapter III: Materials and Methods. Seeds of *Oryza sativa* mutants (for ordered lines see Table 4a-c) were surface sterilized in 70 % ethanol 1 min and 30 min in 3.5 % NaClO followed by three times washing with H<sub>2</sub>O<sub>sterile</sub> 10 min for the propagation of T2 plants. Germination was carried out between two layers of filter paper standing in sterile tap water for 10-14 d at a light intensity of 220 µmol m<sup>-2</sup> s<sup>-1</sup>. Seedlings were transferred in flooded soil from the local campus and grown in the greenhouse at 25-28°C, 16/8 h photoperiod, and 75 % relative humidity. To initiate flowering, plants received short day conditions at 10/14 h photoperiod under the same conditions. The growth cycle from a seedling to grain harvest was 6-9 months depending on the mutant.

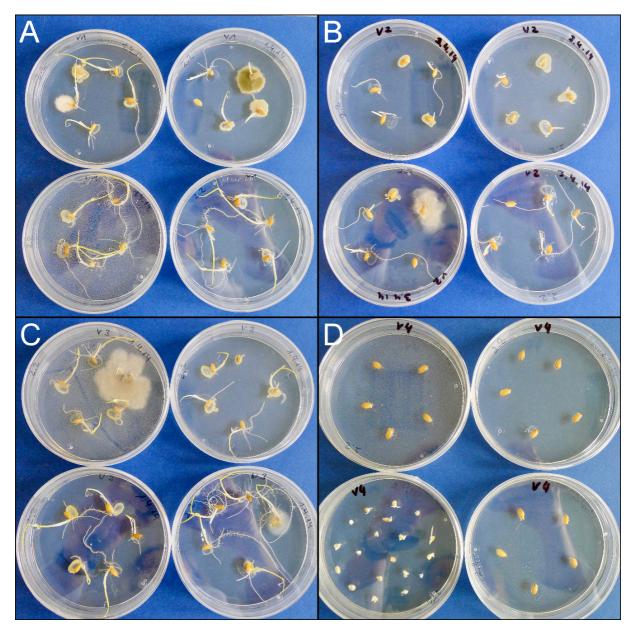
The rice in vitro culture was performed after a modified protocol from Inouge et al. (2013) and Naoko Nishizawa (personal communication). Protocols were varied for seed sterilization, explant preparation, applied antibiotic concentrations, Agrobacterium tumefaciens inoculation, and acclimation. For seed sterilization, four different protocols were tested. First, unhusked seeds were dehydrated using 70% v/v EtOH for 30 s. Afterwards, four different sterilization procedures were tested with different concentrations of NaClO or HgCl<sub>2</sub> and a drop of Tween20 (Fig. 1). Using (A) 3.5% v/v NaClO, 3× H<sub>2</sub>O<sub>sterile</sub> 10 min; (B) 2.6 % v/v NaClO 30 min, 2× H<sub>2</sub>O 20 / 30 min, 2.6 % v/v NaClO 15 min; 3× H<sub>2</sub>O<sub>sterile</sub> 10 min (C) 6 % NaClO 30 min, 3× H<sub>2</sub>O<sub>sterile</sub> 10 min; (D) 3.5 % HgCl<sub>2</sub> 20 min, 3× H<sub>2</sub>O<sub>sterile</sub> 10 min. Solutions used for NaClO were prepared using a 12 % NaClO stock solution. However, only the sterilization with (D) HgCl<sub>2</sub> eliminated all contaminations and was used in all following experiments. Sterilized seeds were then planted on a modified MS culture medium (Supplementary Table S2) for 5-7 d at 28°C at 220 umol m<sup>-2</sup>s<sup>-1</sup> light exposure. Afterwards, the embryos were separated and transferred on N6D callus inducing medium (Supplementary Table S3) and cultured at 28°C for 3-4 weeks. Well grown yellowish, gritty, almost disintegrating calli were transformed along with derivates from b328p6i2xoR-Ucas-U6Os (b328, DNA Cloning Service, Hamburg; Supplementary Fig. S2) using the Agrobacterium tumefaciens strain EHA105. For transformation, calli were collected on filter paper moistened with AAM medium (Supplementary Table S4) and rinsed with the target construct dissolved in 30 ml AAM culture medium to yield 0.1 optical density at 600 nm, and incubated for 10 min. Inoculated calli were then transferred on to 2N6-AS medium (Supplementary Table S3) and co-cultivated at 25°C for 3 days. An overview of all work steps can be found in Table 1.

A Hygromycine B concentration of 50 μg L<sup>-1</sup> was necessary to interrupt the growth of non-transgenic cells (Fig. 2). Culture media were replaced every two weeks until shoot development

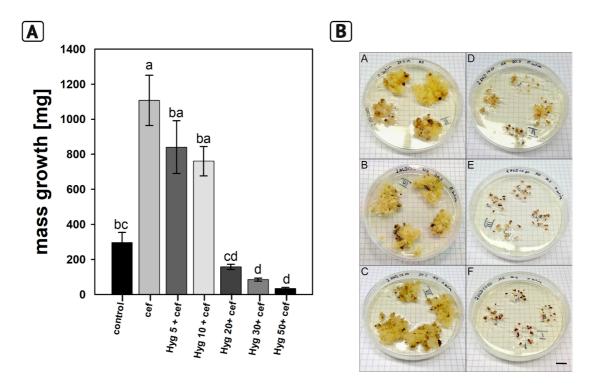
was visible. Shoots were immediately transferred to MSCH50 medium (Supplementary Table S2) for rooting at 28°C in the light. Rooted shoots were transplanted in limed peat substrate (pH 6) at 25-28°C, 16/8 h photoperiod, 100% relative humidity and 220 μmol m<sup>-2</sup>s<sup>-1</sup> with stepwise decreased relative humidity. After acclimation regenerated plantlets were grown in limed peat substrate (pH 6) at 25-28°C, 16/8 h photoperiod, 70 % relative humidity in the greenhouse. Plants were fertilized weekly with a solution containing 0.3 g L<sup>-1</sup> compound fertilizer (18% N, 12% P<sub>2</sub>O<sub>5</sub>, 18% K<sub>2</sub>O, 2% MgO, 6.7% S, 0.1% Fe<sup>EDTA</sup>, 0.05% Mn<sup>EDTA</sup>, 0.04% Cu<sup>EDTA</sup>, 0.02% B, 0.01% Zn<sup>EDTA</sup>, 0.01% Mo<sup>EDTA</sup>). Flowering was induced as described above. An example for successful organogenesis is given in Figure 3.

Table 1: Workflow for rice callus transformation

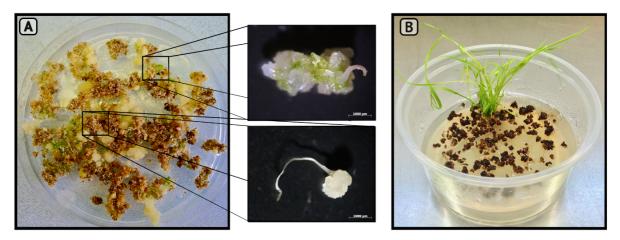
| Days before / after inoculation with<br>Agrobacteria tumefaciens EHA105 | Working step  |
|---|---|
| - 33  | Sterilization of seeds and pre-culture on modified MS culture medium (28°C in the light; Table S2)  |
| - 28  | Embryo preparation and callus induction on N6D culture medium (28°C in the dark; Table S3)  |
| 0   | Transformation/inoculation with <i>A. tumefaciens</i> and co-culture on 2N6-Asmedium (25°C in the dark, Table S3)   |
| 3   | Washing of the callus in MS-culture medium without plant agar containing 500 µg L <sup>-1</sup> Cefotaxime sodium and explanting on 2N6CH10 culture medium (composition see table S1, 28°C in the dark; Table S3) |
| 10  | Transfer of freshly grown yellowish calli on 2N6CH30 culture medium (composition see Table S3, 28°C in the dark)  |
| 17  | Transfer of freshly grown yellowish calli on 2N6CH50 culture medium (composition see Table S3, 28°C in the dark)  |
| 23  | Transfer of freshly grown yellowish calli on MSreCH50 culture medium (composition see Table S2, 8°C in the light)   |
| Starting from 27  | Transfer of developed shoots on MSCH50-culture medium (composition see Table S2)  |



**Figure 1:** Comparison of four different seed surface sterilization methods for *O. sativa* (J) cv. Selenio. All methods included an initial dehydration using 70 % v/v EtOH for 30 s (A) 3.5 % v/v NaClO, 30 min + Tween,  $3 \times H_2O_{steril}$ , 10min; (B) 2.6 % v/v NaClO, 30 min+Tween,  $2 \times H_2O_{steril}$  (20/30 min); 2.6 % v/v NaClO, 15 min + Tween,  $3 \times H_2O_{steril}$ , 10 min; (C) 6 % NaClO v/v, 30 min + Tween,  $3 \times H_2O_{steril}$ , 10min; (D) 2.5 % w/v HgCl<sub>2</sub>+Tween, 20 min;  $3 \times H_2O_{steril}$ , 10 min.



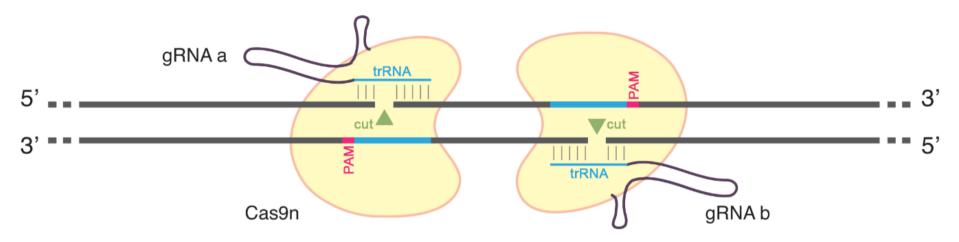
**Figure 2:** Determination of suitable amount of Hygromycine B for selection of transgenic rice cells after 4 weeks of growth. (A) mass growth of different treatments. Control = plants grown on N6D medium; cef = plants grown on N6D + 500  $\mu$ g L<sup>-1</sup> Cefotaxime sodium; Hyg 5/10/20/30/50 + cef = plants grown with 5–50  $\mu$ g L<sup>-1</sup> Hygromycine B and 500 $\mu$ g L<sup>-1</sup> Cefotaxime sodium. Different letters indicate significant differences at a p-value > 0.05 using the tukey-test. (B) phenotype of rice callus; A = cef; B = Hyg5+Cef; C = Hyg10+Cef; D = Hyg20+Cef; E = Hyg30+Cef; F = Hyg50+Cef. Scale bar indicates 1 cm.



**Figure 3:** Organogenesis of rice embryogenic callus. (A) Formation of shoots and roots from embryogenic callus on MSreCH50 culture medium. (B) Plantlets on MSCH50 rooting medium.

## 3.2 Vector design and cloning strategy

The aim of the rice callus transformation was to generate knock out (KO) mutants of the genes of interest. For the generation of KO mutants, a paired nickase vector was generated based on the b328 vector and the results from Fauser et al. (2014). As shown in Figure 4, the paired nickase induces a double nick in the guide RNA (gRNA) in a distance of 3-4 bp (Fauser et al., 2014) from the protospacer adjacent motif (PAM). This introduces a deletion/insertion (Indel) as a result of errors occurring during the non-homologous end joining (Fig. 4; Kirchner et al., 2017; Knoll et al., 2014). The b328 vector is codon optimized for the usage in monocotyledon plants. Furthermore, it carries a double cauliflower mosaic 35s promotor (35s) for expression of the Hygromycine B resistance. The 35s promotor is suitable but not optimal for expression in monocotyledon plants, and it was shown to be less active in root cells (Bicknell and Kultunow, 2004). The nickase is driven by a maize ubiquitin promotor. For functionality of paired nickase, it is necessary to have two different equally expressed gRNAs, in this work a rice U6 (U6os4) promotor sequence was used. For the construction of b328 derivates, the U6 promotor sequence and the second gRNA were amplified from the vector b325pUC19-U6Os4 (b325; Supplementary Fig. 3) using two primers containing a BsmBI motif, specific scaffold sequence (trRNA) and an adapter which binds in b325 (Fig. 5). A list of primers used for scaffold synthesis of respective genes can is shown in Table 1. It was possible to amplify the two site-directed gRNA in the correct orientation by PCR. The amplicon and b328 vector were then digested using BsmBI for site-directed insertion of the amplicon into B328 using a ligase. In the final ΔB328 vector two independent U6 promotors each drive the expression of one guide RNA fused to the scaffold sequence (Fig. 6). An example of the cutting scheme of the used paired nickase is given in Figure 7 using the example of LOC Os06g38930.1. Further cutting schemes for all designed plasmids can be found in Supplementary Table S5. For identification of transgenic plantlets, multiple PCRs were performed. The presence of the marker gene hptII mediating a resistance against Hygromycine B was tested together with the presence of Cas9D10A and the gene  $eF1-\alpha$  in the DNA of T0 regenerated plants from B328:145 and B328:146. Through these PCRs, it was possible to identify (i) the existence of intact plant DNA using eF1-α, (ii) the insertion of hptII, and (iii) insertion of Cas9D10A from ΔB328 in the genomic DNA. The primers and controls used in this approach can be found in Table 3a-b.



**Figure 4:** Schematic figure of double nicking in eukaryotic cells using a paired Cas9D10A modified from Ran *et al.* (2013). Abbreviation: gRNA a/b = respective guide RNA; Cas9n = used nuclease; trRNA = tracking RNA; PAM = protospacer adjacent motif.



Figure 5: Example of a scaffold of LOC\_Os06g38930.1 containing BsmBI motif (green), tracking RNA (trRNA, scaffold of the gRNA) and adapter for binding/cloning in b325pUC19-U6Os4.

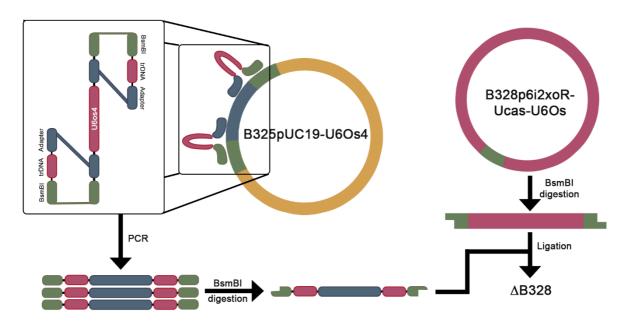


Figure 6: Schematic figure of the cloning strategy for the paired nickase vector b328p6i2xoR-Ucas-U6Os.



**Figure 7:** Schematic overview of gRNA design for the usage of paired nickases using the b328p6i2xoR-Ucas-U6Os vector on the example of LOC\_Os01g63180.1 (B328:145). Abbreviations: NickF=trRNA (scaffold of the gRNA) for 5'-3'-sequence; NickR=trRNA for 3'-5'-sequence; PAM = protospacer adjacent motif; cut = cutting point of respective Cas9D10A.

**Table 2:** Primer sequences used for  $\Delta B328$  construction

| Description           | Sequence   |
|-----------------------|--|
| NickF_144_Os06g38930  | CCCGTCTCCAGCTTGTTGTTACCCTGCAACTGGCAGTTTTAGAGCTAGAAATAGCAAG |
| NickR_144_Os06g38930  | CCCGTCTCAAAACTGTTACCCTGCAACTGGCATCAAGCTGAGCCTCA            |
| NickF_145_Os01g63180  | CCCGTCTCCAGCTTGATCAAGTCAACCAGGGGCATGTTTTAGAGCTAGAAATAGCAAG |
| NickR_145_Os01g63180  | CCCGTCTCAAAACGGGGCATCGGGCAGCTATATCAAGCTGAGCCTCA            |
| NickF_146_ Os07g04970 | CCCGTCTCCAGCTTGCGCGCACGGTGAAGCCATCGGTTTTAGAGCTAGAAATAGCAAG |
| NickR_146_ Os07g04970 | CCCGTCTCAAAACCCGGTGCGCGTGGTGTCGACCAAGCTGAGCCTCA            |
| NickF2_147_Os07g04970 | CCCGTCTCCAGCTTGGATGAGGCTGTCAACTACGAGTTTTAGAGCTAGAAATAGCAAG |
| NickR2_147_Os07g04970 | CCCGTCTCAAAACCTCGATGAGGCTGTCAACTACAAGCTGAGCCTCA            |
| NickF_148_Os01g50100  | CCCGTCTCCAGCTTGCGACGTGCTGATGGTTTGGTTTTAGAGCTAGAAATAGCAAG   |
| NickR_148_Os01g50100  | CCCGTCTCAAAACCCGCCTCGACGTGCTGCTGACAAGCTGAGCCTCA            |

**Table 3a-b:** Primer sequences and control genes used for genotyping of putative transgenic plantlets from rice callus transformation.

Table 3a: Primer sequences for genotyping of T0 plants form rice callus transformation

| Gene               | Orientation | Oligo sequence       |
|--------------------|-------------|----------------------|
| eF1-α              | 5'-3'       | TCAAGTTTGCTGAGCTGGTG |
| (LOC_Os03g08020.1) | 3'-5'       | AAAACGACCAAGAGGAGGGT |
| hptII              | 5'-3'       | GGCTCTGGACGAACTCATGT |
|                    | 3'-5'       | TCAAAGTAGCGCGTTTGCTG |
| Cas9               | 5'-3'       | CCGAGAAGTACAAGGAGATT |
|                    | 3'-5'       | GTACTTCACTTTGGTCAACT |

**Table 3b:** Control genes for genotyping of T0 plants from rice callus transformation

| Gene  | Function  | Amplicon size [bp] |
|-------|---|--------------------|
| eF1-α | Amplicon with WT and T0; no amlicon with B328 & H2O | 164                |
| hptII | Amplicon with B328 and T0, no amplicon with WT      | 391                |
| Cas9  | Amplicon with B328 and T0, no amplicon with WT      | 537                |

In addition to rice callus transformation, T1 seeds with insertions in genes of interests were ordered from the National Institute of Agrobiological Sciences, Japan (tos17; https://tos.nias.affrc.go.jp/index.html.en; Miyao et al., 2003) or from the Crop Biotech Institute, Department of Plant Systems Biotech, Kyung Hee University, Korea (PFG; http://cbi.khu.ac.kr/RISDDB.html; Jeon *et al.*, 2002; An *et al.*, 2003) seed banks providing seeds from T1 plants with insertions from a retrotransposon or T-DNA of *A. tumefaciens*. In the first step, T2 plants were propagated from seeds as described above. Genotyping experiments were carried out to ensure a disruption of the gene of interest as described in Material and Methods of Chapter II, the primer sequences are given in Tables 4a-c.

Table 4a-c: Primer sequences used in genotyping experiments

Table 4a: Primer sequences of mutants for Casparian band biosynthesis-related genes

| Description       | MSU identifier                  | Direction | Sequence              |  |
|-------------------|---------------------------------|-----------|-----------------------|--|
| PFG 3A-17884.R    | PFG 3A-17884.R LOC Os01g46720.1 |           | GTGGCATGGAGCAAATCAGG  |  |
| FFG_5A-17884.R    | LOC_0501g40720.1                | 3'-5'     | TCACCTGCATCAATGAGCATC |  |
| PFG 3D-02260.L    | LOC Os01g63200.1                | 5'-3'     | TCTCTCAGGCAGACATGGAG  |  |
| 11'd_3D-02200.L   | LOC_0501g03200.1                | 3'-5'     | AGAAGATGCTGCTGGAGCTC  |  |
| T46313T (NG2174)  | LOC_Os05g06970.1                | 5'-3'     | TATGAACAGGCCCCAAGAAC  |  |
| 1403131 (1102174) |                                 | 3'-5'     | ACTACGTCGGGCTGACAAAC  |  |
| FL061143(NF2154)  | LOC Os10g09160.1                | 5'-3'     | ATCTGCATCCGTGAGCTCTT  |  |
| FL001143(NF2134)  | LOC_OSTUGU9TUU.T                | 3'-5'     | GGCTCAGAGTGTTTCCAAGC  |  |
| T24092T(NF7704)   | 1.00.00.00.50000.1              | 5'-3'     | AGGTTAAAAATGTGACGGCG  |  |
| T24082T(NF7796)   | LOC_Os02g52830.1                | 3'-5'     | GGAAAGGTGAAAATGCCAGA  |  |

Table 4b: Primer sequences used for the inserted plasmid/retrotransposon

| pGA2715 /-17 (PFG)      | GTTACGTCCTGTAGAAACCCCAA |
|-------------------------|-------------------------|
| pGA2772 (PFG)           | TAGCTAGAGTCGAGAATTCAGT  |
| Retrotransposon (tail6, |                         |
| tos17)                  | AGGTTGCAAGTTAGTTAAGA    |

Table 4c: Primer sequences of mutants for ABC transporter

| Description                   | MSU identifier   | Direction | Sequence              |
|-------------------------------|------------------|-----------|-----------------------|
| PFG 1E-06039.L                | LOC Os02g32690.1 | 5'-3'     | TTCGAAAACTTTCGACTCGAG |
| 11'd_1E-00039.E               | LOC_0502g32090.1 | 3'-5'     | TCGACGTCATCCAACCATAC  |
| PFG_1B-16727.R                | LOC Os09g29660.1 | 5'-3'     | GATCCTGCGAGACTACTGGC  |
| FFG_IB-10/2/.R                | LOC_0809g29000.1 | 3'-5'     | CCCAAGCTGGCTCATCGTTA  |
| PFG 2D-40041.L                | LOC Os02g11760.1 | 5'-3'     | TGCAAGAAGTCTGCAACACC  |
| 11'G_2D-40041.L               | LOC_0302g11700.1 | 3'-5'     | GCAGGGAAACTGGACTCAAC  |
| PFG 3A-06878.R                | LOC_Os08g30740.1 | 5'-3'     | TGCTTGGCTTTTCCAAAATC  |
| FFG_5A-00878.K                | LOC_OS08g30740.1 | 3'-5'     | AGGCAAAACTAAGCTGCAGC  |
| PFG 3A-04171.L                | LOC 0:00:20770 1 | 5'-3'     | TTGACCGCTGATTTCTGTTG  |
| FFO_3A-041/1.L                | LOC_Os08g30770.1 | 3'-5'     | AACCACGAGATCAACAAGCC  |
| PFG 1B-10130.L                | LOC_Os04g13220.1 | 5'-3'     | GGTAGGCCAAGTTGAGCTTG  |
| 11 G_1B-10130.E               |                  | 3'-5'     | AACACGGTATGGAGGGTTTG  |
| NC0475_0_702_1A               | LOC Os08g30770.1 | 5'-3'     | TTTTCAATGCCTTGGGAATC  |
| (NC0475)                      | LOC_0300g30770.1 | 3'-5'     | CCCTCTAAACCCAAAAAGGC  |
| FL038966(NF4005)              | LOC Os01g50100.1 | 5'-3'     | CCAAATACGACGCACATGAC  |
| 12030300(1114003)             | LOC_0301g30100.1 | 3'-5'     | CTATGGCAGGAGAAAGGCAG  |
| ND4056_0_701_1A (ND4056)      | LOC Os08g30740.1 | 5'-3'     | GTAGCTGGGTGAGCGAACTC  |
| 110 1030_0_701_111 (110 1030) | 100_0300830710.1 | 3'-5'     | CATAATTTATTTGCGCGCCT  |
| FL033075 (ND9778)             | LOC_Os02g11760.1 | 5'-3'     | CCCAACGTTCACCAACTTCT  |
| 1 2033073 (1127776)           |                  | 3'-5'     | ATTTTTGTCCGAAAACACGG  |
| FL042012 (NF7561)             | LOC 0-04-12220 1 | 5'-3'     | ATTCCCAAACCCTCCATACC  |
| FL042012 (NF/301)             | LOC_Os04g13220.1 | 3'-5'     | GAGTCGACATGGCTCTCCTC  |

### 4 Results and Discussion

## 4.1 Candidate genes for Si mediated CB development

From the Affymetrix GeneChip experiment, genes with a potential function in CB formation were selected (Table 5). Genes shown in Table 5 were upregulated with a minimum fold change of 1.5 under Si supply in the root section of 2-6 cm behind the root tip. Exodermal CB formation starts 2 cm behind the root tip and ends 8 cm behind the root tip (Fleck *et al.*, 2011). Gene regulation in the harvested root section of 2-6 cm should cover all genes involved in exodermal CB formation. As a control for the GeneChip experiment, it can be shown that the Si transporter Os*Lsi1* and Os*Lsi2* were downregulated with a fold change of 2.83/5.24, respectively, which is in agreement with the literature (Ma *et al.*, 2006, 2007). Genes of interests (GOI) were categorized according to the function in exodermal CB formation as follow: Miscellaneous, phenol metabolism related, transporter, and lipid metabolism. The GOI should cover all aspects of CB formation. To investigate the gene function, KO mutants were produced using a paired nickase or seeds of T1 plants of KO mutants with a retrotransposon or T-DNA insertion (commercially available). Availability of mutants for the respective GOI are marked in blue for paired nickase usage and yellow for ordered T1 mutant seeds.

**Table 5:** Differentially expressed candidate genes related to Casparian band development. Candidates with available constructs for paired nickase investigations are highlighted in blue, present insertion mutants in yellow, p-value is under 0.05.

| Gene assignment | Description | Fold change |
|-----------------|-------------|-------------|
| Miscellaneous   |             |             |

| LOC_Os01g46720.1 | Cyclin-dependent protein serine/threonine kinase activity   | 6.79 |
|------------------|---|------|
| LOC_Os06g38930.1 | Leucine-rich repeat receptor protein kinase EXS precursor   | 7.61 |
| LOC_Os02g28130.1 | Fasciclin like arabinogalactan protein                      | 3.61 |
| LOC_Os08g29020.1 | Wall-associated kinase-like 2, putative, expressed          | 2.81 |
| LOC_Os06g38830.1 | Receptor-like protein kinase precursor, putative, expressed | 2.41 |
| LOC_Os04g23700.1 | Lectin protein kinase family protein, putative, expressed   | 1.88 |

## Phenol metabolism-related

| LOC_Os04g24140.1 | Enzyme of the glycolysis; in connection with shikimate-<br>synthesis of phenylalanine | 2.21 |
|------------------|---|------|
| LOC_Os12g39860.2 | Enzyme of the glycolysis; in connection with shikimate-<br>synthesis of phenylalanine | 2.02 |
| LOC_Os01g63180.1 | Laccase-6 precursor; Lignin degradation and detoxification                            | 2.28 |
| LOC_Os07g04970.1 | N-hydroxycinnamoyl benzoyltransferase-like protein                                    | 2.99 |

| LOC_Os03g58890.2 | Oxidoreductase, putative, expressed  | 2.37 |
|------------------|--|------|
| LOC_Os05g06970.1 | Peroxidase precursor, induces phenol oxidation   | 2.19 |
| LOC_Os01g63200.1 | Laccase precursor protein, putative, expressed;<br>Cupredoxin domain containing protein. | 1.77 |
| LOC_Os03g55410.1 | Peroxidase precursor, putative, expressed  | 1.76 |
| LOC_Os06g12250.1 | Sphingolipid C4-hydroxylase SUR2, putative, expressed                                    | 1.71 |
| LOC_Os01g44260.1 | Dihydroflavonol-4-reductase, putative, expressed   | 1.70 |

## Gene assignment Description

## Fold change

## Transporter

| LOC_Os02g32690.2 | Pleiotropic drug resistance protein 15, putative, expressed | 1.71 |
|------------------|---|------|
| LOC_Os08g30770.1 | ABC transporter, ATP-binding protein, putative, expressed   | 2.12 |
| LOC_Os04g13220.1 | ABC transporter family protein, putative, expressed         | 1.88 |
| LOC_Os01g50100.1 | ABC transporter, ATP-binding protein, putative, expressed   | 1.69 |
| LOC_Os08g30740.1 | ABC transporter, ATP-binding protein, putative, expressed   | 1.65 |
| LOC_Os09g29660.1 | White-brown complex homolog protein 11, putative, expressed | 1.70 |
| LOC_Os02g11760.1 | Pleiotropic drug resistance protein, putative, expressed    | 1.69 |

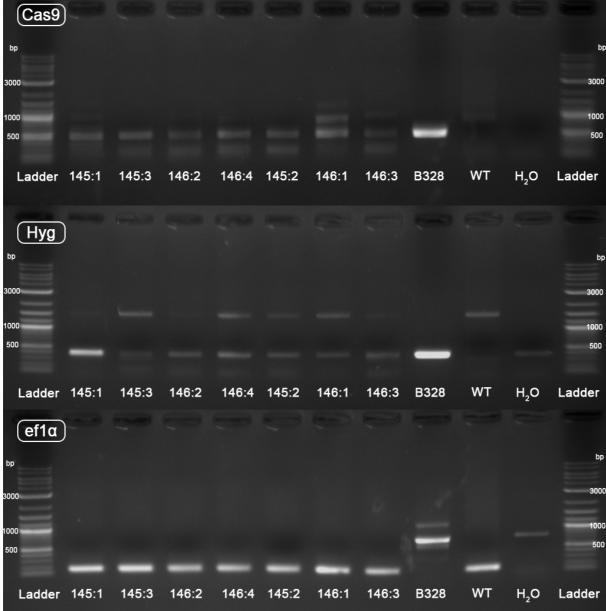
## Lipid metabolism-related

| LOC_Os06g39780.1 | Cytochrome P450, putative, expressed; entcassadiene C11-alpha-hydroxylase 1  | 3.01 |
|------------------|--|------|
| LOC_Os03g07060.1 | Glycerol-3-phosphate acyltransferase, putative                               | 1.91 |
| LOC_Os10g09160.1 | Cytochrome P450, putative, expressed   | 1.81 |
| LOC_Os05g40384.1 | Cytochrome P450, putative, expressed   | 1.62 |
| LOC_Os07g23710.1 | Cytochrome P450, putative  | 1.56 |
| LOC_Os06g30500.1 | Cytochrome P450, putative, expressed   | 1.56 |
| LOC_Os11g17580.1 | Enoyl-CoA hydratase/isomerase family protein                                 | 1.53 |
| LOC_Os02g52830.1 | Lipase, putative, expressed  | 1.53 |
| LOC_Os04g20880.1 | Wax synthase isoform 1, putative, expressed                                  | 1.53 |
| LOC_Os09g16520.1 | Cytochrome b5-like Heme/Steroid binding domain containing protein, expressed | 1.51 |

#### 4.2 Rice callus transformation

The transformation of rice callus was necessary due to the lack of suitable KO mutants. For KOs, a paired nickase was used (B328; Supplementary Fig. S2) and plasmids were generated for LOC Os06g38930.1 (B328:144) a "leucine-rich-repeat receptor protein kinase EXS precursor" probably involved in Si mediated signal cascade; LOC Os01g63180.1 (B328:145) "laccase-6-precursor" which could be involved in formation of aromatic compounds for CB LOC Os07g04970.1 (B328:146; B328:147) "N-hydroxycinnamovl formation and benzoyltransferase-like protein" which is most probably involved in biosynthesis of suberin/lignin for CB formation; LOC Os01g50100 (B328:148) "ABC transporter", which could be involved in CB compound transport. After successful cloning of respective B328 derivates, the constructs were transformed into rice callus using the A. tumefaciens mediated gene transfer. It was not possible to regenerate plants from LOC Os06g38930.1 (B328:144) and LOC Os07g04970.1 (B328:147). The reason for this effect is unknown for B328:147. For B328:144 an overlap of the the PAM sequence and the gRNA scaffold by 1 nt caused a functional deficiency of this construct (Supplementary Table S5). Because of this failure, the paired nickase was probably not able to target the PAM sequence and no nick was induced. The construct LOC Os01g50100 (B328:148) was not transformed into rice callus because of time limitations. The sequential sequences of the successfully cloned  $\Delta B328$  can be found in Supplementary Table S6.

The success of the transformations was checked by PCR (Fig. 8). All T0 plants and the wildtype (WT) showed an amplicon for  $eF1-\alpha$ . Furthermore, Hygromycine B (hptII) and Cas9D10A (Cas9) showed an amplicon with the correct size of ~400 / ~500 bp together with the positive control of B328 DNA template (Fig. 8). It is possible that the amplicons in the H<sub>2</sub>O control using  $eF1-\alpha$  and hptII primers originated from a contamination. However, this is negligible, since there is no amplicon in the H<sub>2</sub>O control and WT using Cas9 primers, the transgenicity is proven. B328 showed also multiple amplicons using  $eF1-\alpha$  primers because of unspecific binding in the vector. In T0 plants multiple amplicons were visible using Cas9- and hptII primers which could be explained by intron-splicing in the plant and unspecific fragments. In summary, the experiments showed evidence for the transgenicity of tested T0 plants. However, it is an open question, whether these T0 plants are cut by paired nickases and if the respective sequences are modified by Cas9. In theory, paired nickases should be able to interrupt all copies of the genes of interests in the genome. Until now this was not found for paired nickase (J. Geike, Institute of Plant Genetics, Molecular Plant Breeding, personal communication) but it was shown that Cas9 can induce homozygous plants in T0 (Brooks et al., 2014).



**Figure 8:** PCR of putative transgene T0 plants resulting from rice callus transformation with B328:145 and B328:146. Upper part: Usage of Cas9 primers for Cas9D10A detection; Middle part: Usage of primer against the Hygromycin B resistance gene (*hptII*). Lower part: Usage of primers against eF1-α. Abbreviations: Ladder = 2-Log DNA Ladder (0.1-10.0 kb; New England Biolabs); 145:1-3/146:1-4 = for PCR used DNA template origins from rice callus transformation with the construct B328:145/B328:146, single plants are numerized starting from 1; B328 = for PCR used DNA template was b328p6i2xoR-Ucas-U6Os; WT = for PCR used DNA template was DNA from *O. sative (L.)* cv. Selenio.

### 4.3 Transposon/T-DNA insertion Lines

To investigate GOI from the Affymetrix Genechip experiment (Table 5), T1 seeds were ordered from PFG or *Tos17* seed banks. The seeds were a mixture of homozygous transgenic-/wildtype-and heterozygous plants. Thus, it was necessary to find homozygous transgenic plants using genotyping PCRs. In general, genotyping of plants from PFG was more difficult than *Tos17* because of unspecific binding of the given genotyping primers. Furthermore, seeds of PFG had a very poor germination rate (around 10%, data not shown). For this reason, the owners of the

PFG seed bank do not supply seeds to Europe anymore (Sook An, personal communication). A list of all tested mutants with the testing results can be found in Tables 6a-b. It was only possible to find 7 homozygous transgenic T1 lines out of 16 lines.

**Table 6a-b:** Overview from ordered T-DNA and retrotransposon insertion lines with their results in genotyping. **Table 6a**: Casparian band biosynthesis related genes.

| <b>Identifier (Database)</b> | MSU Identifier   | Gene Assignment   | Database | Wildtype   | Genotyping  |
|------------------------------|------------------|---|----------|------------|-------------|
| PFG_3A-17884.R               | LOC_Os01g46720.1 | Cyclin-dependent protein serine/threonine kinase activity | PFG      | Hwayoung   | heterozygot |
| PFG_3D-02260.L               | LOC_Os01g63200.1 | Laccase precursor protein                                 | PFG      | Dongjin    | homozygot   |
| T46313T (NG2174)             | LOC_Os05g06970.1 | Peroxidase precursor, putative, expressed                 | Tos17    | Nipponbare | heterozygot |
| FL061143(NF2154)             | LOC_Os10g09160.1 | Cytochrome P450   | Tos17    | Nipponbare | homozygot   |
| T24082T(NF7796)              | LOC_Os02g52830.1 | Lipase, putative, expressed                               | Tos17    | Nipponbare | homozygot   |

## Table 6b: ABC Transporter

| <b>Identifier (Database)</b> | MSU Identifier   | Gene Assignment   | Databse | Wildtype   | Genotyping  |
|------------------------------|------------------|---|---------|------------|-------------|
| PFG_1E-06039.L               | LOC_Os02g32690.2 | Pleiotropic drug resistance protein 15, putative, expressed | PFG     | Dongjin    | ?           |
| PFG_1B-16727.R               | LOC_Os09g29660.1 | White-brown complex homolog protein 11                      | PFG     | Dongjin    | heterozygot |
| PFG_2D-40041.L               | LOC_Os02g11760.1 | Pleiotropic drug resistance protein, putative, expressed    | PFG     | Dongjin    | heterozygot |
| PFG_3A-06878.R               | LOC_Os08g30740.1 | ABC transporter   | PFG     | Dongjin    | heterozygot |
| PFG_3A-04171.L               | LOC_Os08g30770.1 | ABC transporter, ATP-binding protein, putative, expressed   | PFG     | Dongjin    | homozygot   |
| PFG_1B-10130.L               | LOC_Os04g13220.1 | ABC transporter family protein, putative, expressed         | PFG     | Dongjin    | homozygot   |
| NC0475_0_702_1A (NC0475)     | LOC_Os08g30770.1 | ABC transporter, ATP-binding protein, putative, expressed   | Tos17   | Nipponbare | heterozygot |
| FL038966(NF4005)             | LOC_Os01g50100.1 | ABC transporter, ATP-binding protein, putative, expressed   | Tos17   | Nipponbare | heterozygot |
| ND4056_0_701_1A (ND4056)     | LOC_Os08g30740.1 | ABC transporter, ATP-binding protein, putative, expressed   | Tos17   | Nipponbare | heterozygot |
| FL033075 (ND9778)            | LOC_Os02g11760.1 | Pleiotropic drug resistance protein, putative, expressed    | Tos17   | Nipponbare | homozygot   |
| FL042012 (NF7561)            | LOC_Os04g13220.1 | ABC transporter family protein, putative, expressed         | Tos17   | Nipponbare | homozygot   |

## General discussion

Silicon has beneficial effects on plants, especially on those species, which are able to accumulate Si, such as gramineous plants. Several effects like an enhanced mechanical stability of shoots, induced resistance against powdery mildew, higher resistance against herbivores or enhanced drought resistance are well described in the literature (for review see Epstein, 1999). It is further known that silicic acid supply reduces As uptake in O. sativa (L.) which was first discussed by Guo et al. (2007). This was later explained by the reduced expression of the Si transporters OsLsi1 and OsLsi2 which also transport arsenite (Ma and Yamaji 2008). Furthermore, it was observed that Si supply enhances the CB formation in the exodermis of rice, which reduces the ROL. The Si enhanced formation of CB was not limited to rice but occurred also in roots of Zea maize, Allium cepa, Tradescantia virginiana, and Guizotia abvssinica (Fleck et al., 2015; Chapter I). To investigate the molecular background of the stimulated CB formation, transcriptomic techniques were used such as a custom-made microarray (Chapter II), qPCR (Chapters I+II+III) and whole genome GeneChips (Chapters III+IV). The custom-made microarray was designed using only genes related to CB development, such as phenylpropanoid-pathway related genes or lipid metabolism related genes (Fleck et al., 2011). As a result, it was possible to identify 19 genes upregulated through Si fertilization in rice roots. The results of the microarray triggered further investigations in OsABCG25 (Chapter II). Because of the limited number of genes on the custom-mademicroarray, an Affymetrix GeneChip hybridization was performed, covering most transcripts in rice, to get genome-wide insight into the transcriptome of Si treated rice roots. These investigations additionally revealed more genes, which are potentially involved in CB formation (Chapter IV). Surprisingly, an upregulation of Fe homeostasis related genes was also found (Chapter III).

### **Evaluation of Casparian band staining**

The CB in onion, maize, and rice consists of aliphatic and aromatic components such as lignin and suberin in well-developed exodermal CB (Chapter I, Supplementary Fig. 5). Due to staining of serial sections, it was possible to show that Fluorol Yellow 088 and Berberin-Anilin stains the same compartments in onion, maize and rice roots. Fluorol Yellow 088 stains aliphatic suberin lamellae (Brundrett *et al.*, 1988) and Berberin-Anilin indicates aromatic compounds in suberin. Unlike the endodermal CB in *Arabidopsis thaliana*, the suberin lamellae are built from

the very beginning of CB deposition in the exodermis (Perumalla and Peterson, 1986; Enstone and Peterson, 1997;).

For staining with Fluorol Yellow 088, the procedure in Brundrett *et al.* (1991) was modified because no staining was visible using the given protocol which uses Fluorol Yellow 088 in polyethylene glycol. Instead, the usage of heated lactic acid as a solvent for Fluorol Yellow 088 was suitable for staining as described by Lux *et al.* (2005). Lactic acid is a better solvent for hydrophobic dyes because of its hydrophobicity and protonic properties. The lactic acid can dissolve hydrophobic substances upon heating and enhances the possibility that the hydrophobic substances bind to the hydrophobic compartments in the tissue caused by the hydrophobic interaction entropic advantage. The 'hydrophobic interaction entropic advantage' describes how hydrophobic substances enhance the reaction with other hydrophobic substances in the presence of protonic acids like lactic acid and other hydrophilic substances. During the evaluation of the Fluorol Yellow 088 stained tissues, it was observed that fading occurs after around 20 s in higher magnifications using a GFP filter cassette which agrees with findings of Brundrett *et al.* (1991).

## ABC transporter and Casparian band development

OsABCG25 is most probably responsible for transport of suberin monomers (Chapter II; Fig. 6). The evidence of the substrate specificity is based on transcriptomic results. The 4-coumarate ligase (4CL) was found to be downregulated by OE of Os*ABCG25* which is indicating a transfer of monolignol precursors (p-coumaroyl-CoA or coumaric acid) into suberin monomers instead of secretion for lignin polymerisation. According to the findings, the diacyglycerol O-acytransferase (DGOAT) was found to be highly upregulated and is therefore suggested to incorporate aliphatic components for suberin monomer formation. The standard errors of the GC-FID measurements were quite high which could overlay differences. The experiments need to be repeated with a higher number of replications for estimation of changes in suberin composition.

Another ABC transporter, the OsABCG32 has already been characterized as a transporter for aliphatic cutin (Garroum *et al.*, 2016), involved in the formation of cuticular layers in leaves indicating the impact of OsABCG transporter for the release of aliphatic substances from the epidermal/exodermal cell layers into the apoplast of shoots and roots.

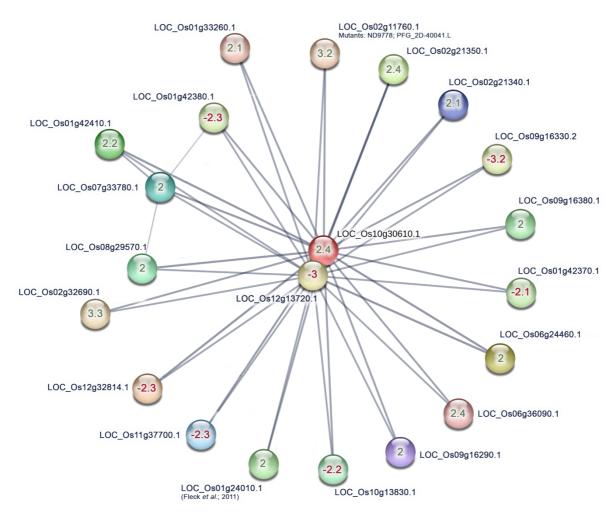
In our experiments with *osabcg25*, a downregulation of the respective transcript was observed but without different phenotype. It is possible that OsABCG25 is acting as either a homodimer or heterodimer, and its function might be compensated by another ABCG transporter. It is

shown in the literature that ABCG transporter indeed can be a homodimer or heterodimer (for review on ABC transporters see Verrier *et al.*, 2008).

An analysis of the predicted protein interaction using STRING software (Szklarczyk et al., 2015) revealed 19 possible interaction partners (Fig. 1). They share possible equal functions and protein structures with a confidence level over 0.6 (low: <0.4; high: >0.6). The dataanalysis based on a prediction of experimental data, database annotation, homology, coexpression and automated text mining. All predictions together result in a confidence level score. A list of all ABC-motif containing loci, which are possible interaction partners with OsABCG25 can be found in Table 1. The analysis provides evidence for the hypothesis that a lack of OsABCG25 due to a KO is compensated by another interaction partner. It is shown that seven putative interaction partners were downregulated under Si treatment (Fig. 1). A downregulation in the root tissue could indicate an involvement in lignin transport since lignin production in the CB development seems to be reduced based on the results given in Chapter II. LOC Os12g13720.1 has the same interaction partner as OsABCG25. It would be interesting if the LOC Os12g13720.1 could be the corresponding lignin monomer transporter, which is downregulated by the OE of OsABCG25. Furthermore, the LOC Os12g13720.1 could be the missing transporter for substitution of OsABCG25 due to its expression pattern. The expression pattern of LOC Os12g13720.1 was not tested in the KO and OE mutant of OsABCG25. Twelve ABC-motif containing genes were upregulated in the Si treated roots. Thus, these genes can be hypothesized to possibly substitute the function of OsABCG25 in osabcg25 and are coexpressed together with OsABCG25 for a heterodimer formation in WT and OE conditions together with OsABCG25. One ABC transporter was already observed by Fleck et al. (2011) but without further results because of a lack of suitable mutants. For OsABCG39 (LOC Os02g11760.1) two homozygote KO mutants (ND9778 & PFG 2D-40041.L) were generated in this work (Chapter IV: Results) without further functional analysis. For the splicing variant of OsABCG41 1 (LOC Os02g32690.2), the KO mutant PFG 1E-06039.L is available as homozygote mutant.

 Table 1: List of possible interaction partner with OsABCG25 (LOC\_Os10g30610.1).

| <b>Identifier</b> | Gene assignment & Publications                             |
|-------------------|--|
| LOC_Os01g24010.1  | Os <i>ABCG32</i>   |
| LOC_Os01g33260.1  | Os <i>ABCG33</i>   |
| LOC_Os01g42370.1  | Os <i>ABCG35</i>   |
| LOC_Os01g42380.1  | Os <i>ABCG36</i> ; Park <i>et al.</i> (2009)               |
| LOC_Os01g42410.1  | Os <i>ABCG37</i>   |
| LOC_Os02g11760.1  | Os <i>ABCG39</i>   |
| LOC_Os02g21340.1  | Os <i>ABCG40</i>   |
| LOC_Os02g21350.1  | ABC-2 type transporter family protein, expressed           |
| LOC_Os02g32690.2  | Os <i>ABCG41_2</i>   |
| LOC_Os06g24460.1  | multidrug transporter (ABCG/PDR-type)                      |
| LOC_Os06g36090.1  | Os <i>ABCG42_1</i> ; Zhang <i>et al.</i> (2016)            |
| LOC_Os07g33780.1  | Os <i>ABCG43</i> ; Zhang <i>et al.</i> (2016)              |
| LOC_Os08g29570.1  | Os <i>ABCG44</i>   |
| LOC_Os09g16290.1  | Os <i>ABCG46</i>   |
| LOC_Os09g16330.2  | Os <i>ABCG53</i>   |
| LOC_Os09g16380.1  | Os <i>ABCG47</i>   |
| LOC_Os10g13830.1  | Os <i>ABCG51</i>   |
| LOC_Os11g37700.1  | Os <i>ABCG48</i>   |
| LOC_Os12g32814.1  | pleiotropic drug resistance protein 3, putative, expressed |



**Figure 1:** Predicted protein interactions using STRING (Szklarczyk *et al.*, 2015). The bubbles indicate possible interaction partner, the connection line shows the confidence of the data which is considered high at a value of 0.7. Number in the bubbles represent the respective regulation of the gene in Si treated roots from the Gene Chip experiment in Chapters III+IV.

In rice roots, not only ABCG transporters are regulated by Si, but also ABCA, -B, and -C transporters. All ABC transporter have one or more transmembrane-motif(s) and one or more nucleotide binding domain(s) (Saha *et al.*, 2015). In this work, it was possible to identify homozygous mutants of other members of the ABC transporter families OsABCA, OsABCB, OsABCC and OsABCG (Chapter IV; Table 1). Two homozygous KO mutants for OsABCA (OsABCA3 (LOC\_Os08g30740.1) and OsABCA4 (LOC\_Os08g30770.1) were identified. For the OsABCB family, the OsABCB5 (LOC\_Os01g50100.1) was identified, while for the OsABCC family the gene OsABCC10 (LOC\_Os04g13220.1) was found. As another member of the OsABCG family, a homozygote mutant for OsABCG22 (LOC\_Os09g29660.1) was determined. ABC transporters are localized in the membrane of many plant cell types and organelles (for review see Kang *et al.*, 2011) but localization and function of the identified ABC transporters are unknown.

## Influence of phenol metabolism in CB development

The CB in rice consists of suberin and lignin (Schreiber et al., 2005b; Naseer et al., 2012). Monomers of suberin and lignin are secreted into the apoplast where they condense to polymers and build the CB. In the endodermis of Arabidopsis thaliana, lignin builds a diffusion barrier first (Naseer et al., 2012) and later the deposition of suberin takes place (for review see Doblas et al., 2017). Lignin consists of aromatic components such as ferulic acid or coumaric acid while suberin is a more aliphatic polymer made out of  $\omega$ -hydroxy acids, fatty acids and alcohols connected with aromatic backbones such as ferulic acid. These precursors are provided by the phenylpropanoid pathway and from the fatty acid metabolism (Chapter II, Fig. 6). Enzymes catalyzing reactions in these two pathways should be upregulated in rice roots grown in Si containing nutrient solution since the CB formation is enhanced due to Si treatment. For some genes probably involved in the phenol metabolism homozygous mutants were generated: three mutants for the "Laccase-6 precursor" (LOC\_Os01g63180.1 & LOC\_Os01g63200.1, paired nickase using the plasmid b328:146 or b328:147, mutant: PFG 3D-02260.L), one mutant for the "N-hydroxycinnamoyl benzoyltransferase like protein" (LOC Os07g04970.1; plasmid: b328:147) and one mutant for a peroxidase (LOC Os05g06970.1, mutant: NG2174. The "Laccase-6 precursor" has a predicted function in lignin degradation and detoxification of lignin-derived products in the apoplast and thus could be involved in polymerization of monolignols (Lu et al., 2013; Rico et al., 2014) and in this way in CB formation.

The "N-hydroxycinnamoyl benzoyltransferase like protein" was also found upregulated due to As treatment in roots in transcriptomic studies but no functional interpretation was provided (Norton *et al.*,2008). Expression increase of the "N-hydroxycinnamoyl benzoyltransferase like protein" was in the same extent as the expression due to Si treatment.

Peroxidases might mediate the formation of radicals. The found peroxidase (LOC\_Os05g06970.1) is predicted to be involved in the oxidation of phenols to phenolic radicals in the apoplast (Doğan *et al.*, 2016). A String analysis revealed a possible interaction with three dehydrogenases (cinnamoyl alcohol dehydrogenase 8A, LOC\_Os09g23530.1, Tobias and Chow, 2005; cinnamoyl alcohol dehydrogenase 6, LOC\_Os04g15920.1, Tobias and Chow, 2005; cinnamoyl alcohol dehydrogenase 8B, LOC\_Os09g23540.1) which all are predicted to be involved in lignin biosynthesis and lignin monomer formation out of coniferyl-, sinapyl- or 4-coumarate alcohol. This peroxidase could be involved in multiple reactions in lignin polymer formation in the apoplast after release of lignin monomers and could be highly interesting for further investigations.

### Impact of fatty acids in exodermal CB formation

Further components of CB are fatty acids for the formation of suberin monomers. Fatty acid oxidation provides aliphatic C-chains for the suberin formation. With regard to the fatty acid metabolism, the homozygous KO mutants for LOC\_Os10g09160.1 (Cytochrome P450, mutant: NF2154) and LOC\_Os02g52830.1 (lipase class 3-like, mutant: NF7796) were found. Cytrochrome P450 is involved in the oxidative degradation of aliphatic fatty acid chains and possibly interacts with several aldehyde oxidases such as LOC\_Os07g18154.1 according to String analysis. The aldehyde oxidases could be responsible for further metabolic processes. The yet uncharacterized lipase (LOC\_Os02g5230.1) is known to be related to the lipid metabolic process with a predicted hydrolase activity.

One KO mutant is available for the Leucine-rich repeat receptor protein kinase EXS precursor (LOC\_Os06g38930.1, mutant: PFG\_3D-02260.L) which is yet uncharacterized. Its function was described as a potential protein kinase with no experimental data. Using String analysis, several carboxylases, chaperons, and phosphatases were predicted to interact with LOC\_Os06g38930.1. These predicted interactions suggest a function in protein modification and due to the nature of leucine rich repeats, a participation of the leucine-rich repeat as the start of a signal cascade can be anticipated.

## Dirigent proteins and their influence in CB development

In the literature, it is shown that laccases and peroxidases themselves are not able to mediate the formation of lignin and suberin from precursors, since this would only result in racemic coupling (Davin *et al.*, 1997). The oxidative reaction of these phenols needs to be guided, which is suggested to be mediated by dirigent proteins. Dirigent proteins are small proteins (~80 kD) without any catalytic active oxidative center. They seem to bind only one substrate and have a characteristic signal peptide. Without dirigent proteins, the coupling of aliphatic or aromatic compounds would not occur stereo selectively and the products would be unspecific. The first of these dirigent proteins was found in *Forsythia intermedia* (psd\_Fi7; Gang *et al.*, 1999) and in *A. thaliana* the dirigent protein 'ectopic suberin1' (ESB1) has been already characterized (Hosmani *et al.*, 2013). In *A. thaliana*, ESB1 is responsible for the correct formation of suberin lamellae, thus a lack of ESB1 results in randomly organized CB. These findings in *A. thaliana* underline the importance of dirigent proteins in CB formation in endodermal CB. The role of dirigent proteins in exodermal CB formation is unclear. According to the Affymetrix GeneChip data (Chapter III), 18 dirigent proteins out of 35 were found upregulated through Si in rice roots (Table 2) Thus, these genes could be involved in the Si dependent formation of exodermal CB.

**Table 2:** List of possible dirigent protein coding genes regulated by Si in roots of *O. sativa* (L.) with a minimal p-value of 0.05.

| Identifier     | Gene assignment                                   | Foldchange |
|----------------|---|------------|
| LOC_Os11g07770 | Dirigent, putative, expressed                     | 4.07       |
| LOC_Os11g07740 | Dirigent, putative                                | 2.55       |
| LOC_Os11g07710 | Dirigent, putative                                | 2.52       |
| LOC_Os07g01660 | Dirigent, putative, expressed                     | 2.51       |
| LOC_Os11g40180 | Dirigent, putative, expressed                     | 2.39       |
| LOC_Os07g44450 | Dirigent, putative, expressed                     | 2.28       |
| LOC_Os01g26340 | Dirigent, putative                                | 2.25       |
| LOC_Os11g07680 | Dirigent, putative, expressed                     | 2.19       |
| LOC_Os11g07690 | Dirigent, putative                                | 2.16       |
| LOC_Os11g07670 | Dirigent, putative, expressed                     | 2.16       |
| LOC_Os03g05030 | Dirigent, putative, expressed                     | 2.13       |
| LOC_Os12g09720 | Dirigent, putative                                | 2.12       |
| LOC_Os07g01680 | Dirigent, putative                                | 2.12       |
| LOC_Os11g27620 | Dirigent, putative                                | 2.08       |
| LOC_Os10g25900 | Dirigent, putative, expressed                     | 2.07       |
| LOC_Os11g10850 | Dirigent, putative                                | 2.05       |
| LOC_Os03g17210 | expressed protein                                 | 2.05       |
| LOC_Os04g57130 | Dirigent-like protein pDIR17, putative, expressed | 2.00       |
| LOC_Os07g42570 | Dirigent, putative                                | -2.01      |
| LOC_Os11g10870 | Dirigent, putative, expressed                     | -2.02      |
| LOC_Os07g44370 | Dirigent, putative, expressed                     | -2.05      |
| LOC_Os07g44930 | Dirigent, putative, expressed                     | -2.05      |
| LOC_Os08g26180 | Dirigent, putative                                | -2.05      |
| LOC_Os12g07580 | Dirigent, putative, expressed                     | -2.06      |
| LOC_Os10g25870 | Dirigent, putative, expressed                     | -2.07      |
| LOC_Os07g44380 | Dirigent, putative, expressed                     | -2.10      |
| LOC_Os01g24960 | Dirigent, putative                                | -2.11      |
| LOC_Os08g26560 | Dirigent, putative                                | -2.12      |
| LOC_Os11g42500 | Dirigent, putative, expressed                     | -2.20      |
| LOC_Os07g42520 | Dirigent, putative, expressed                     | -2.30      |
| LOC_Os12g12600 | Dirigent, putative, expressed                     | -2.41      |
| LOC_Os11g42550 | Dirigent, putative, expressed                     | -2.59      |
| LOC_Os11g07830 | Dirigent, putative, expressed                     | -3.41      |
| LOC_Os01g25030 | Dirigent, putative, expressed                     | -4.55      |
| LOC_Os12g26380 | Dirigent, putative, expressed                     | -20.29     |

## Influence of Si in nutrient acquisition in rice

Silicon somehow reduces the Fe concentration in the leaves of rice, which was shown in several experiments in optimal and excess Fe conditions (Ma and Takahashi, 1990; Ma *et al.*, 2001; Dufey *et al.*, 2013). In these studies, it was also shown that Si supply reduced the concentration

of As, Al, Ca, Mg, Mn and P reduced by Si. The model in chapter III describes that the Si dependent enhanced formation of exodermal CB hampers the flux of Fe from the rhizosphere into the apoplast and thus reduces the uptake of Fe (Chapter III; Figure 7). This effect could also occur for other elements, where apoplastic transport is thought to play a significant role in uptake such as Ca and Mg. Following this suggestion, elements that are taken up symplastically should be not altered in their uptake due to Si, which is true in case of potassium (Dufey et al., 2013). Mn cannot be directly explained by the model described in this work. Manganese is taken up in the plant as Mn<sup>2+</sup> and is known to be reduced (Mn<sup>2+</sup>) or oxidized (Mn<sup>4+</sup>) by microorganisms in the rhizosphere / apoplast (Kothari et al., 1991). Additionally, exodermal CB reduces the ROL and induce an oxygen shortage in the rhizosphere, which could inhibit bacteria growth and thereby availability of Mn species. The reduced uptake of As can be explained by the expression pattern of the two Si transporter OsLsi1 and OsLsi2. Their expression levels are decreased in rice due to Si. Both transporters are able to transport As in form of arsenite (Ma et al., 2007; Mitani et al., 2009a). The abundance of Si reduces the amount of transporter and in this way the subsequent uptake of As, too. For Al, it was indicated by Ma et al. (2001) that Si forms Al-Si complexes in the anaerobic soil solution which are insoluble and not taken up by the plant. Furthermore, Si treatment enhances the secretion of phenols in presence of Al. It is suggested that phenols can bind Al and detoxify it in the rhizosphere (Kidd et al., 2001). In addition, the earlier formation of CB due to Si might also hamper the flux of Al into the apoplast and reduce the uptake of Al in this way. The reasons for the reduced uptake of anorganic P remain unclear. It is discussed that the reduced uptake of other ions could influence the internal availability of P (Ma and Takahashi, 1990).

## **Outlook**

## Functional analysis of candidate genes

Several homozygous KO mutants for the Si dependent exodermal CB formation were obtained, however, a functional analysis is not included yet (Chapter IV). For these mutants the respective transcript level has to be determined by qPCR and the CB phenotype has to be investigated by Fluorol Yellow 088 or Berberin-Anilin-staining. Furthermore, mutants should be characterized by ROL visualization and nutrient uptake as described in Chapter II for OsABCG25. For high throughput screening of the ROL it could be considered to use planar optodes as described in Larsen et al. (2015). Planar optodes have the advantage to directly investigate the O<sub>2</sub> flux in the root rhizosphere under natural conditions with many samples synchronously. Additionally, suitable OE mutants are necessary since KO mutants may have no different phenotype because of their substitution. For the generation of OE mutants full transgenic rice lines are necessary by means of a suitable binary plasmids like pIPKb002 (Himmelbach et al., 2007). The use of a maize ubiquitin promotor is crucial in rice for a suitable OE of the candidate gene (Kumlehn, Leibniz Institute of Plant Genetics and Crop Plant Research, personal communication). Additionally, it would be useful to determine the substrate for all ABC transporter, which could be done by in vitro expression of respective genes using oocytes of Xenopus laevis as described in Nozoye et al. (2011).

## **Functional analysis of OsABCG25**

OsABCG25 was suggested to transport suberin. A String analysis revealed many possible interacting ABC transporters of OsABCG25. In transcriptomic studies their interaction could be verified by single qPCR measurements or a whole genome Affymetrix GeneChip. An additional Affymetrix GeneChip experiment might be used to investigate the transcriptome of the KO and OE of OsABCG25 to reveal new interaction partners, in order to gain new insights in possible reaction pathways.

It should be further considered to investigate the impact of OsABCG49 (LOC\_Os12g13720.1) in *osabcg25*. Due to the String analysis, OsABCG49 was found to have the same interaction partners as OsABCG25. Os*ABCG49* was downregulated in roots by Si treatment while OsABCG25 was upregulated. Os*ABCG25* is presumed to be a suberin transporter due to its regulation in the OE. A double KO of OsABCG49 in *osabcg25* would be helpful to investigate the exact gene function of Os*ABCG25* and Os*ABCG49*.

### **Dirigent proteins in exodermal CB formation**

Dirigent proteins are mandatory for the guided oxidative coupling of suberin monomers. The function of dirigent proteins in Si dependent CB development was not yet considered for selection of candidate genes. This is promising, since the results from the whole genome Affymetrix GeneChip indicate that 18 out of 35 genes coding for a dirigent protein were upregulated in the roots of rice due to Si treatment.

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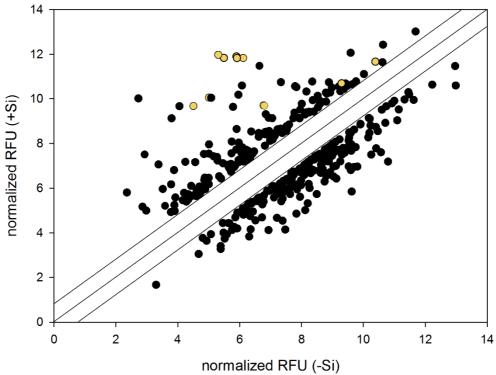
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# **Supplementary Material**

# **Supplementary Material: Chapter III**



**Supplementary Figure S1:** Scatterplot of genes up- or downregulated through Si-treatment. The lines indicate the minimum fold change cut-off of 1.5 in the data; yellow colored dots indicate Fe-related genes. Data are normalized relative fluorescence units (RFU) from the Affymetrix Genechip experiment with a p-value cut-off of 0.01.

Supplementary Table S1: List of used primers for qPCR analysis.

UBQ5

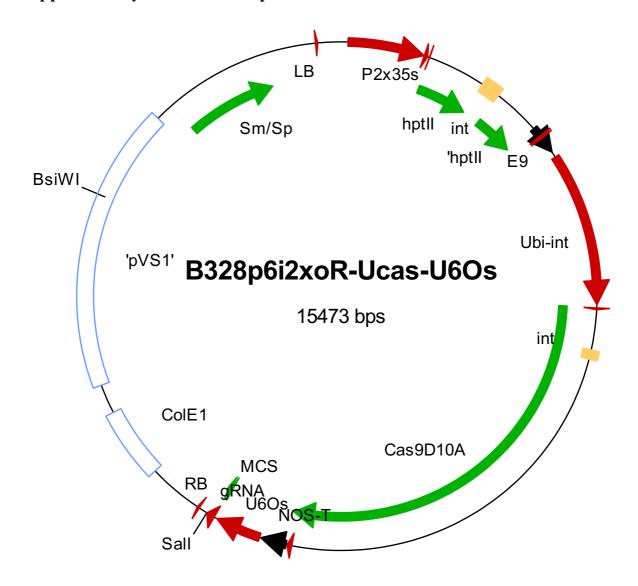
LOC\_Os01g22490

| Gene assignment      | Locus                | Orientation    | Oligo sequence                               |
|----------------------|----------------------|----------------|--|
|                      |                      | 5'-3'          | GCCGCATCCGCAGCGGAAGATCA                      |
| Os <i>DMAS</i>       | LOC_Os03g13390.2     | 3'-5'          | CTCTCTCTCGCACGTGCTAGCGT                      |
|                      |                      | 5'-3'          | TACTTCATTTCAGAACAAGC                         |
| Os <i>ENA1</i>       | LOC_Os11g05390.1     | 3'-5'          | GCATTTGGCTTCTGGCTTAA                         |
| 0. 737.44            |                      | 5'-3'          | TTCGTCTTCCTCTCGACCTCTC                       |
| Os <i>ENA2</i>       | LOC_Os06g48060.1     | 3'-5'          | TCCCTGACATCATGCTCTGAAGC                      |
| 0 10 001             | LOC_Os08g01090       | 5'-3'          | ATGGACGACATGGTGCTCC                          |
| Os <i>IDEF1</i>      |                      | 3'-5'          | CTAGGGATTTGTTGTCTGCT                         |
| 0. 10.002            | LOC_Os05g35170.1     | 5'-3'          | TCTCTGCTGGTTTCTCAAAGGATG                     |
| Os <i>IDEF2</i>      |                      | 3'-5'          | GCAGCAAGGGAAACATTTCAGC                       |
| 0.1002               | 1.00.0.01.70070.1    | 5'-3'          | CACCAGCACCACCAACTGCAAACCAG                   |
| Os <i>IRO2</i>       | LOC_Os01g72370.1     | 3'-5'          | GCTTTGTTCCTGACGACTTTCTCC                     |
| O-11D71              | 1 0 0 0 0 1 10 170 2 | 5'-3'          | GAATTCCACAAATGCCGGGAGAAAGG                   |
| Os <i>HRZ1</i>       | LOC_Os01g49470.2     | 3'-5'          | AGCCAGCAAGGCGTCCAA                           |
| $O_2NAAT1$           | LOC Os02g20360.1     | 5'-3'          | TAAGAGGATAATTGATTTGCTTAC                     |
| Os <i>NAAT1</i>      | LOC_OS02g20300.1     | 3'-5'          | CTGATCATTCCAATCCTAGTACAAT                    |
| $O_{0}NASI$          | LOC Os03g19427.1     | 5'-3'          | GTCTAACAGCCGGACGATCGAAAGG                    |
| Os <i>NAS1</i>       | LOC_0803g19427.1     | 3'-5'          | TTTCTCACTGTCATACACAGATGGC                    |
| Os <i>NAS2</i>       | I OC 0202~10420.2    | 5'-3'          | TGAGTGCGTGCATAGTAATCCTGGC                    |
| OSIVA52              | LOC_Os03g19420.2     | 3'-5'          | CAGACGGTCACAAACACCTCTTGC                     |
| Os <i>NAS3</i>       | LOC Os07g48980.1     | 5'-3'          | CGATCGAGTGTTCGACTGATCACC                     |
| OSIVADJ              | LOC_0307g40700.1     | 3'-5'          | TCCACCGTCATACTCTCTCTCTCG                     |
| Os <i>TOM1</i>       | LOC_Os11g04020.1     | 5'-3'          | GCCCAAGAACGCCAAAATGA                         |
| Os <i>I OM I</i>     |                      | 3'-5'          | GGCTTGAAGGTCAACGCAAG                         |
| Os <i>TOM2</i>       | LOC_Os11g04030.1     | 5'-3'          | GGCGCAGGTGTTCTATTCTCTTGG                     |
|                      |                      | 3'-5'          | ACCGCCAAGAAAGGTTTGAAGGTC                     |
| Os <i>TOM3</i>       | LOC Os07g01250.1     | 5'-3'          | TGGAGGTAATGCAACGTCCC                         |
| 0310103              | 200_030/g01230.1     | 3'-5'          | AAAGACGAGAGCACGGACTG                         |
| Os <i>12g0132500</i> | Os12g0132500         | 5'-3'          | GGCGGTGTTCTATGATGGCT                         |
| 081280132300         |                      | 3'-5'          | ACTCCTTGTAGGGGATGCCT                         |
| Os <i>YSL13</i>      | LOC Os04g44300.1     | 5'-3'          | TGCAACTGCCTTCCTCATAAACGG                     |
| 0010210              |                      | 3'-5'          | ACCGAGTTTCTTTACTTGCTTCGC                     |
| Os <i>YSL2</i>       | LOC Os02g43370.2     | 5'-3'          | GAGGACAACGGTGTCATTGCTGGT                     |
| 031002               |                      | 3'-5'          | TGCAGAAAAGCCCTCGACGCCAAGA                    |
| Os <i>YSL6</i>       | LOC Os04g32050.1     | 5'-3'          | TGTTGCACTGCGCAAGGTAATG                       |
|                      | 0                    | 3'-5'          | TGTCGCAGTTCCACTGGGATAG                       |
| Os <i>YSL8</i>       | LOC Os02g02460.1     | 5'-3'          | TGGGATGCATAATAAGCCCGATGG                     |
|                      |                      | 3'-5'          | CATCCGATGTTGTTCGCCTTG                        |
| Os <i>YSL15</i>      | LOC_Os02g43410.2     | 5'-3'<br>3'-5' | GGCTATTAGCTTCGCGGTA                          |
| Os <i>YSL16</i>      | LOC_Os04g45900.1     | 5'-3'          | CTCCTTGGGCTGTGTGGAAC<br>GGTGGATTCGGGTCGTTCTT |
|                      |                      | 3'-5'          | ATGACCAGCACCTTTCGGAG                         |
| Housekeeping G       | enes                 | 1 3-3          | INTORCOUCACCITICUUAU                         |
|                      | LOC_Os03g08020.1     | 5'-3'          | TCAAGTTTGCTGAGCTGGTG                         |
| eF1-α                |                      | 3'-5'          | AAAACGACCAAGAGGAGGGT                         |
| T.D.O.=              |                      | 5'-3'          | ACCACTTCGACCGCCACTACT                        |
| UBQ5                 | LOC_Os01g22490       | 21.51          | A GOOGET A A GOOTE OF COTTE                  |

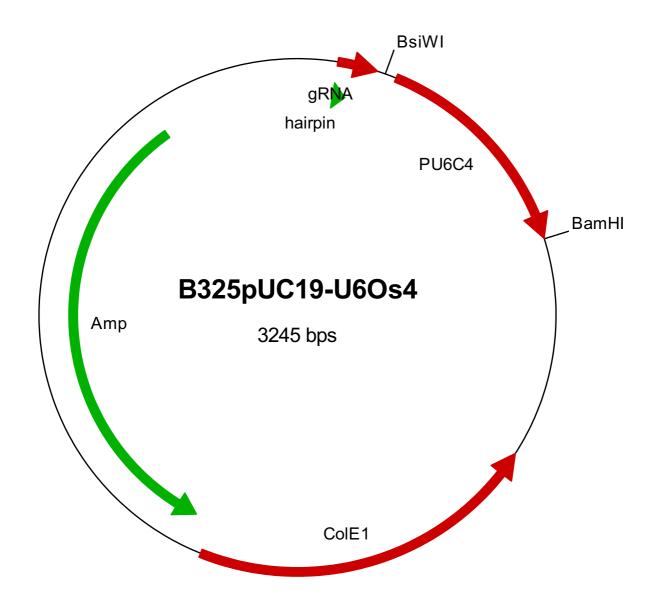
3'-5'

ACGCCTAAGCCTGCTGGTT

# Supplementary Material: Chapter IV



**Supplementary Figure 2:** Vector map of the binary vector B328p6i2xoR-Ucas-U6O carrying Hygromycine B resistance gene (hptII) and the paired nickase (Cas9D10A) between left- (LB) and right boarder (RB) for integration into the plant genome via *Agrobacteria tumefaciens* mediated gene transfer. Abbreviations: ColE1 = origin of replication; pVS1= origin of replication for *A. tumefaciens*; Sm/Sp = bacterial streptomycin/spectomycin resistance; LB = left boarder; P2x35s = double cauliflower mosaic 35s promotor; hptII = Hygromycine B resistance; int = intron; E9 = terminator; Ubi-Int = maize ubiquitin promotor; Cas9D10A = modified Cas9 nuclease; NOS-T = nopalinsynthase terminator; U6Os = U6 promotor for rice; gRNA = guide RNA; MCS = multiple cloning site; RB = right boarder.



**Supplementary Figure 3:** Vector map of B325pUC19-U6Os4 for amplification of the site directed scaffold carrying respectively gRNAs and PAM sequences. ColE1 = origin of replication; Amp = ampicillin resistance; hairpin = recognition site for binding of Cas9n; gRNA = guide RNA, carrying PAM and trRNA for respectively gene; PU6C4 = U6 promotor for rice.

**Supplementary Table S2:** Culture-medium composition for shoot (MSreCH50) and root regeneration (MSCH50) of transformed rice calli.

| Substance                         | MSreCH50                            | MSCH50                                    | modified MS                              |
|-----------------------------------|-------------------------------------|---|--|
| KNO <sub>3</sub>                  | 1.9 g L <sup>-1</sup>               | 1.9 g L <sup>-1</sup>                     | 1.9 g L <sup>-1</sup>                    |
| $CaCl_2 \times 2 H_2O$            | 442 mg L <sup>-1</sup>              | 442 mg L <sup>-1</sup>                    | 442 mg L <sup>-1</sup>                   |
| $MgSO_4 \times 7 H_2O$            | 370 mg L <sup>-1</sup>              | 370 mg L <sup>-1</sup>                    | 370 mg L <sup>-1</sup>                   |
| KH <sub>2</sub> PO <sub>4</sub>   | 170 mg L <sup>-1</sup>              | 170 mg L <sup>-1</sup>                    | 170 mg L <sup>-1</sup>                   |
| $H_3BO_3$                         | 12.4 mg L <sup>-1</sup>             | 12.4 mg L <sup>-1</sup>                   | 12.4 mg L <sup>-1</sup>                  |
| $MnSO_4 \times 4H_2O$             | $0.045 \text{ mg L}^{-1}$           | $0.045 \text{ mg L}^{-1}$                 | 0.045 mg L <sup>-1</sup>                 |
| $ZnSO_4 \times 7H_2O$             | 17.2 mg L <sup>-1</sup>             | 17.2 mg L <sup>-1</sup>                   | 17.2 mg L <sup>-1</sup>                  |
| KI                                | 1.66 mg L <sup>-1</sup>             | 1.66 mg L <sup>-1</sup>                   | 1.66 mg L <sup>-1</sup>                  |
| $Na_2MoO_4 \times 2 H_2O$         | 0.5 mg L <sup>-1</sup>              | 0.5 mg L <sup>-1</sup>                    | $0.5~\mathrm{mg}~\mathrm{L}^{\text{-}1}$ |
| $CuSO_4 \times 5 H_2O$            | $0.05~\mathrm{mg~L^{-1}}$           | $0.05~\mathrm{mg~L^{-1}}$                 | 0.05 mg L <sup>-1</sup>                  |
| $CoCl_2 \times 6 H_2O$            | $0.05~{\rm mg}~{\rm L}^{\text{-}1}$ | $0.05~\mathrm{mg}~\mathrm{L}^{\text{-}1}$ | $0.05~\mathrm{mg~L^{\text{-}1}}$         |
| $FE^{EDTA}$                       | $0.096~{ m mg}~{ m L}^{-1}$         | $0.096~{ m mg}~{ m L}^{-1}$               | 0.096 mg L <sup>-1</sup>                 |
| Thiamin × HCl                     | 1 mg L <sup>-1</sup>                | 1 mg L <sup>-1</sup>                      | 1 mg L <sup>-1</sup>                     |
| Niacin                            | 5 mg L <sup>-1</sup>                | 5 mg L <sup>-1</sup>                      | 5 mg L <sup>-1</sup>                     |
| Pyrodoxin × HCl                   | 5 mg L <sup>-1</sup>                | 5 mg L <sup>-1</sup>                      | 5 mg L <sup>-1</sup>                     |
| Glycin                            | 20 mg L <sup>-1</sup>               | 20 mg L <sup>-1</sup>                     | 20 mg L <sup>-1</sup>                    |
| Sorbitol                          | 30 g L <sup>-1</sup>                |   |  |
| Glucose                           |                                     |   | 2 g L <sup>-1</sup>                      |
| Saccharose                        | 30 g L <sup>-1</sup>                | 30 g L <sup>-1</sup>                      | 30 g L <sup>-1</sup>                     |
| Prolin                            | $2.8~{ m g}~{ m L}^{\text{-1}}$     |   |  |
| Casamino Acid                     | 2 g L <sup>-1</sup>                 |   |  |
| MES                               | 1.1 g L <sup>-1</sup>               |   |  |
| Myo-Isonitol                      | 100 mg L <sup>-1</sup>              | 100 mg L <sup>-1</sup>                    | 100 mg L <sup>-1</sup>                   |
| Gelrite <sup>™</sup>              | 4 g L <sup>-1</sup>                 |   | 4 g L <sup>-1</sup>                      |
| Plant agar                        |                                     | 8 g L <sup>-1</sup>                       |  |
| 1-Naphthaleneacetic acid (NAA)    | 2 mg L <sup>-1</sup>                |   |  |
| Kinetin                           | 1 mg L <sup>-1</sup>                |   |  |
| Cefotaxime sodium                 | 500 mg L <sup>-1</sup>              | $500 \text{ mg L}^{-1}$                   |  |
| Hygromycine B                     | 50 mg L <sup>-1</sup>               | 50 mg L <sup>-1</sup>                     |  |
| pH = 5.8 adjusted with 1 M KOH or | 1 M H <sub>2</sub> SO <sub>4</sub>  |   |  |

**Supplementary Table S3:** Culture-medium composition for callus induction (N6D), callus-co-cultivation (2N6-As), and callus regeneration medium (2N6CH10/30/50)

| Substance                       | N6D                     | 2N6-As                       | 2N6CH10/30/50                             |
|---------------------------------|-------------------------|------------------------------|---|
| NH <sub>4</sub> SO <sub>4</sub> | 463 mg L <sup>-1</sup>  | 463 mg L <sup>-1</sup>       | 463 mg L <sup>-1</sup>                    |
| KNO <sub>3</sub>                | 2.831 g L <sup>-1</sup> | 2.831 g L <sup>-1</sup>      | 2.831 g L <sup>-1</sup>                   |
| $CaCl_2 \times 2 H_2O$          | 166 mg L <sup>-1</sup>  | 166 mg L <sup>-1</sup>       | 166 mg L <sup>-1</sup>                    |
| $MgSO_4 \times 7 H_2O$          | 185 mg L <sup>-1</sup>  | 185 mg L <sup>-1</sup>       | 185 mg L <sup>-1</sup>                    |
| KH <sub>2</sub> PO <sub>4</sub> | 400 mg L <sup>-1</sup>  | 400 mg L <sup>-1</sup>       | 400 mg L <sup>-1</sup>                    |
| H <sub>3</sub> BO <sub>3</sub>  | 1.6 mg L <sup>-1</sup>  | 1.6 mg L <sup>-1</sup>       | 1.6 mg L <sup>-1</sup>                    |
| $MnSO_4 \times 4H_2O$           | 4.4 mg L <sup>-1</sup>  | 4.4 mg L <sup>-1</sup>       | 4.4 mg L <sup>-1</sup>                    |
| $ZnSO_4 \times 7H_2O$           | 1.5 mg L <sup>-1</sup>  | 1.5 mg L <sup>-1</sup>       | 1.5 mg L <sup>-1</sup>                    |
| KI                              | 0.8 mg L <sup>-1</sup>  | 0.8 mg L <sup>-1</sup>       | 0.8 mg L <sup>-1</sup>                    |
| Fe <sup>EDTA</sup>              | 9.64 mg L <sup>-1</sup> | 9.64 mg L <sup>-1</sup>      | 9.64 mg L <sup>-1</sup>                   |
| Thiamine × HCl                  | 10 mg L <sup>-1</sup>   | 10 mg L <sup>-1</sup>        | $10~\mathrm{mg}~\mathrm{L}^{\text{-}1}$   |
| Niacin                          | 5 mg L <sup>-1</sup>    | 5 mg L <sup>-1</sup>         | 5 mg L <sup>-1</sup>                      |
| Pyrodoxine × HCl                | 5 mg L <sup>-1</sup>    | 5 mg L <sup>-1</sup>         | 5 mg L <sup>-1</sup>                      |
| Glycine                         | 20 mg L <sup>-1</sup>   | 20 mg L <sup>-1</sup>        | $20 \text{ mg L}^{-1}$                    |
| Proline                         | 2.8 g L <sup>-1</sup>   |                              | 2.8 g L <sup>-1</sup>                     |
| Saccharose                      | 30 g L <sup>-1</sup>    | 30 g L <sup>-1</sup>         | 30 g L <sup>-1</sup>                      |
| Glucose                         |                         | 10 g L <sup>-1</sup>         |   |
| Casamino acid                   |                         | 1 g L <sup>-1</sup>          | 1 g L <sup>-1</sup>                       |
| Gelrite <sup>TM</sup>           | 4 g L <sup>-1</sup>     | 4 g L <sup>-1</sup>          | 4 g L <sup>-1</sup>                       |
| 2,4-Dichlorophenoxyacetic acid  | 2 mg L <sup>-1</sup>    | 2 mg L <sup>-1</sup>         | 2 mg L <sup>-1</sup>                      |
| Hygromycine B                   |                         |                              | 500 μg L <sup>-1</sup>                    |
| Cefotaxime sodium               |                         |                              | $10 / 30 / 50~\mu g~L^{-1}$               |
| Acetosyringone                  |                         | 20 mg L <sup>-1</sup> (added |   |
|                                 |                         | directly prior               |   |
|                                 |                         | use)                         |   |
| pH (2N6-As / N6D, 2N6CH10/30    | 0/50) = 5.2 / 5.8       | adjusted with 1 M K          | COH or 1 M H <sub>2</sub> SO <sub>4</sub> |

# Supplementary Table S4: Medium for Agrobacteria inoculation (AAM-Medium)

| Substance                                      | Concentration                                    |
|--|--|
| $CaCl_2 \times 2 H_2O$                         | 150 mg L <sup>-1</sup>                           |
| $MgSO_4 \times 7 H_2O$                         | 250 mg L <sup>-1</sup>                           |
| KH <sub>2</sub> PO <sub>4</sub>                | 169.6 mg L <sup>-1</sup>                         |
| KCl  | 2950 mg L <sup>-1</sup>                          |
| $H_3BO_3$                                      | 3 mg L <sup>-1</sup>                             |
| MnSO <sub>4</sub> × 1 H <sub>2</sub> O         | 7.02 mg L <sup>-1</sup>                          |
| $ZnSO_4 \times 7 H_2O$                         | 2 mg L <sup>-1</sup>                             |
| KJ   | 0.75 mg L <sup>-1</sup>                          |
| $Na_2MoO_4 \times 2 H_2O$                      | 0.025 mg L <sup>-1</sup>                         |
| $CuSO_4 \times 5 H_2O$                         | 0.25 mg L <sup>-1</sup>                          |
| $CoCl_2 \times 6 H_2O$                         | 0.025 mg L <sup>-1</sup>                         |
| Fe <sup>EDTA</sup>                             | 16.84 mg L <sup>-1</sup>                         |
| Glutamine                                      | 876 mg L <sup>-1</sup>                           |
| Aspartic acid                                  | 266 mg L <sup>-1</sup>                           |
| Arginine                                       | 174 mg L <sup>-1</sup>                           |
| Glycine  | $7.5 \text{ mg L}^{-1}$                          |
| Myo- Inositol                                  | 100 mg L <sup>-1</sup>                           |
| Niacin   | 1 mg L <sup>-1</sup>                             |
| Thiamine - HCl                                 | 10 mg L <sup>-1</sup>                            |
| Pyridoxine - HCl                               | 1 mg L <sup>-1</sup>                             |
| Saccharose                                     | 68.5 g L <sup>-1</sup>                           |
| Glucose  | 36 g L <sup>-1</sup>                             |
| Casamino Acid                                  | 1 g L <sup>-1</sup>                              |
| Acetosyringone                                 | 20 mg L <sup>-1</sup> (added directly prior use) |
| $pH = 5.2$ adjusted with 1 M KOH or 1 M $H_2S$ | O <sub>4</sub>                                   |

Supplementary Table S5: Cutting schemes of paired nickase vectors based on b328p6i2xoR-Ucas-U6Os

# LOC Os06g38930.1 (B328:144)

#### gRNA 1

TTGTTACCCTGCAACTGGCAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGC

#### gRNA 2

TGTTACCCTGCAACTGGCATGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGC

### Cut-scheme

# LOC\_Os07g04970.1 (B328:146)

#### gRNA 1

CGCGCACGGTGAAGCCATCGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGC

#### gRNA 2

CCGGTGCGCGTGTCGACGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGC

#### Cut- Scheme



# LOC\_Os07g04970.1 (B328:147)

# gRNA 1

GATGAGGCTGTCAACTACGAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAG GCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGC

# gRNA 2

CTCGATGAGGCTGTCAACTAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGC

# Cut- Scheme

# LOC Os01g50100.1 (B328:148)

#### gRNA 1

CGACGTGCTGATGGTTGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGC

## gRNA 2

CCGCCTCGACGTGCTGAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGC

|  | PAM sequence $\Downarrow$ |
|--|---------------------------|
| NickF  | >                         |
|  | cut                       |
| CGACG  | TGCTGCTGATGGTTG           |
| GGGGAAGAAGGTGCCGCTGTTCAGCTTGTTCCGGTACG <mark>CCG</mark> ACCGCCTCGACG | TGCTGCTGATGGTTGTC A       |
|  | TGCTGCTGA                 |
| cut  |                           |
| PAM sequence ↑ <   | NickR                     |

#### **Supplementary Table S6:** Sequential sequences for ΔB328 constructs

#### **Description** Sequence

|          | ATTGTCGTTTCCCGCCTTCGGTTTAAACTATCAGTGTTTGACAGGATATATTGGCG |
|----------|--|
| B328:144 | GGTAAACCTAAGAGAAAAGAGCGTTTATTAGAATAATCGGATATTTAAAAGGGCGT |
|          | GAAAAGGTTTATCCGTTCGTCCATTTGTATGTGCATGCCAACCACAGGGTTCCCCT |
|          | CGGGAGTGCTTGGCATTCCGTGCGATAATGACTTCTGTTCAACCACCCAAACGTCG |
|          | GAAAGCCTGACGACGGAGCAGCATTCCAAAAAGATCCCTTGGCTCGTCTGGGTCGG |
|          | CTAGAAGGTCGAGTGGGCTGCTGTGGCTTGATCCCTCAACGCGGTCGCGGACGTAG |
|          | CGCAGCGCCGAAAAATCCTCGATCGCAAATCCGACGCTGTCGAAAAGCGTGATCTG |
|          | CTTGTCGCTCTTTCGGCCGACGTCCTGGCCAGTCATCACGCGCCAAAGTTCCGTCA |
|          | CAGGATGATCTGGCGCGAGTTGCTGGATCTCGCCTTCAATCCGGGTCTGTGGCGGG |
|          | AACTCCACGAAAATATCCGAACGCAGCAAGATATCGCGGTGCATCTCGGTCTTGCC |
|          | TGGGCAGTCGCCGCCGACGCCGTTGATGTGGACGCCGAAAAGGATCTAGGTGAAGA |
|          | TCCTTTTTGATAATCTCATGACCAAAATCCCTTAACGTAAGTTTTCGTTCCACTGA |
|          | GCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTCTGCG |

CGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAG ATACCAAATACTGTTCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTC TGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCCCA GTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAG GCGCAGCGGTCGGGCTGAACGGGGGGGTTCGTGCACACAGCCCCAGCTTGGRAGCG AACGACCTACACCGGAACTGAA TcGGnngccTTTTtcaGTTGataCGGACTAGCCTTATTTtAaCTTGCTATTTCTAGCTCTAAA  ${\tt ACGGGGCATCGGGCAGCTATATCAAGCTGAGCCTCAGCGCAGCAGCTTATAAGCAAGTGGTCG}$  ${\tt CGTCGTtGCGTGAGCGAGGTGGTACTAAACTGTTGTTTCCGCCCACGCGCTCTGCAATCCCAC}$  ${\tt CGTCCACGGGGCAAGGGCCACTTGTGGGCCGGACCTCTAGCTGGATCTCCCCACCACGGAGA}$ AAAGTGGGCCGTGGCCTGTGGGCCCCCTCATTTCTTTCCCTTTTGTATTGCTGGGCCGACTGGA GGGAATTTGGTCCATTGTACTGAGAGTCATATGCCCATTTCTTAGAAATTGTTCATTTGTCCC ATATTACAAGTCCCTCTTTCTCAAAAGGTGTCATGTAAAAAATAATACGTTCCCTAATTAAAA TTTTTATAGTAAAGAATATTTTTTAAATTCTGGGTTTACATGATTATCGTATCATCTCTCCGG GAAGAAAAGAAAACGTACCTACGTAATGCTGTACAGCGTAATGCCAACTTTCGTACGAGAAA B328:145 GCTGGGTCTAAAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTT ATTTTAACTTGCTATTTCTAGCTCTAAAACATGCCCCTGGTTGACTTGATCAAGCTGAGCCTC  ${\tt AGCGCAGCTTATAAGCAAGTGGTCGCGTCGTTGCGTGAGCGAGGTGGTACTAAACTGTTG}$  $\tt CTCTAGCTGGATCTCCCCACCACGGAGAAAnGTGGGCCGTGGGCCTGTGGGCCCCTCATTTCT$  $\tt TTCCCTTTGTATTGCTGGGCCGACTGGAGGGAATTTGGTCCATTGTACTGAGAGTCATATGCC$ CATTTCTTAGAAATTGTTCATTTGTCCCATATTACAAGTCCCTCTTTCTCAAAAGGTGTCATG TAAAAAnTAATACGTTCCCTAATTAAAnTTTTTTATAGTAAnGAAtATTTTTAAAATTCtGGgT  ${\tt TtACATGATTATcgnatcAtctctCcgggAanAAaannAAAaCGTACCTACGTAATgnt}$  ${\tt CtcGGntqCccTTTTTttcaGTTtGaTAnnCGGACTAGCCTTATTTTAACTTGCTATTTCTAG}$  $\tt CTCTAAAACCCGGTGCGCGTGGTGTCGACCAAGCTGAGCCTCAGCGCAGCAGCTTATAAGCAA$  ${\tt GTGGTCGCGTCGTTGCGTGAGCGAGGTGGTACTAAACTGTTGTTTCCGCCCACGCGCTCTGCA}$ ATCCCACCGTCCCACGGGGCAAGGGCCACTTGTGGGCCGGACCTCTAGCTGGATCTCCCCACC GACTGGAGGGAATTTGGTCCATTGTACTGAGAGTCATATGCCCATTTCTTAGAAATTGTTCAT TTGTCCCATATTACAAGTCCCTCTTTCTCAAAAGGTGTCATGTAAAAAATAATACGTTCCCTA ATTAAAATTTTTATAGTAAAGAATATTTTTTAAATTCTGGGTTTACATGATTATCGTATCATC TCTCCGGGAAGAAAAGAAAACGTACCTACGTAATGCTGTACAGCGTAATGCCAACTTTCGTA  $\tt CGAGAAAGCTGGGTCTAAAAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGAC$ B328:146 GAGCCTCAGCGCAGCAGCTTATAAGCAAGTGGTCGCGTCGTTGCGTGAGCGAGGTGGTACTAA ACTGTTGTTTCCGCCCACGCGCTCTGCAATCCCACCGTCCCACGGGGCCAAGGGCCACTTGTGG GCCGGACCTCTAGCTGGATCTCCCCACCACGGAGAAAnGTGGGCGTGGCCTGTGGGCCCC CTCATTTCTTTCCcTTTGTATTGCTGGGCCGACTGGaGGgAATTTGGTCCATTGTACTGAGAGTCATATGCCCATTTCTT  ${\tt AGAAATTGTTCATTTGTCCCATATTACAAGTCCcTCTTTCTCAAAnGgtgTCATGTAAAAant}$  $\verb|aaTACGTTCCCTAATTAAAATTTTtATAGtAangAanaTTtttnAAATtCTGggTtnACaGAT| \\$  ${\tt TATCgnatCATcnctccggnaananaagAAAnCgtaCCTACgtaatGctgnac}$ CncGGntqccTTTTtcaGTTtGaTtaCGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCT AAAACCTCGATGAGGCTGTCAACTACAAGCTGAGCCTCAGCGCAGCAGCTTATAAGCAAGTGG  ${\tt CACCGTCCCACGGGGCAAGGGCCACTTGTGGGCCGGaCcTCTAGCTGGATCTCCCCACCACGGGCACCTCTAGCTGGATCTCCCCACCACGGGCACGGGCCACGGGGCCACGGGGCCACGGGCCACGGGGCCGGGCCACGGGCCACGGGGGCCACGGGGCCACGGGGCCACGGGGCCACGGGGCCACGGGGCCACGGGGCACGGGGCCACGGGGCACGGGGCACGGGGCACGGGGCACGGGGCACGGGGCACGGGGCACGGGGCACGGGGCCACGGGGCACGGGGCACGGGGGCACGGGGCCACGGGGCCACGGGGCCACGGGGCCACGGGGCCACGGGGCCACGGGGGCCACGGGGCCACGGGGCCACGGGGGCCACGGGGCCACGGGGCCA$  ${\tt agAAAAGTGGGCCTTGTGGGCCCCTCATTTCTTTCCCTTTGTATTGCTGGGCCGACT}$  ${\tt GGAGGGAATTTGGTCCATTGTACTGAGAGTCATATGCCCATTTCTTAGAAATTGTTCATTTGT}$ CCCATATTACAAGTCCCTCTTTCTCAAAAGGTGTCATGTAAAAAATAATACGTTCCCTAATTA CGGGAAGAAAAGAAAACGTACCTACGTAATGCTGTACAGCGTAATGCCAACTTTCGTACGAG  ${\tt AAAGCTGGGTCTAAAAAAAaGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGC}$  $\tt CTTATTTTAACTTGCTATTTCTAGCTCTAAAACTCGTAGTTGACAGCCTCATCCAAGCTGAGC$ CTCAGCGCAGCAGCTTATAAGCAAGTGGTCGCGTCGTTGCGTGAGCGAGGTGGTACTAAACTG B328:147 TTGTTTCCGCCCACGCGCTCTGCAATCCCACCGTCCCACGGGGCAAGGGCCACTTGTGGGCCG  ${\tt GACCTCTAGCTGGATCTCCCcACCACGGaqAAAaGTGGGCCGTGGCCTGTGGqCCCCCTCATT}$  ${\tt TCTTTCCcTTTGTATTGCTGGGCCGACTGGAGGGAATTtGGTCCATTGTACTGAGAGTCATAT}$ GCCCATTTCTTAGAAnTTGTTCATTTGTCCCATATTACAAGTCccTCTTTCTCAAAAGqtqTC ATqtAaaAanTAATACGTTCCCTAATtAAAnTTTTTTATAqnnaaqaataTTTTttAAaTtCTG GnTTnACatGATTATCGtatcAnntCTCcgggAAgAaangAAanCGTACCTACGTAATgctgn ncGaaTtct

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 $a {\tt CncGgtgccTTTTtcaGTTgaTanCGGACTAGCCTTATTTtAACTTGCTATTTCTAGCTCTA}$  $\verb|AAACCCGCCTCGACGTGCTGACAAGCTGAGCCTCAGCGCAGCAGCTTATAAGCAAGTGGT|$  $\tt CGCGTCGTTGCGTGAGCGAggTGGTACTAAACTGTTGTTTCCGCCCACGCGCTCTGCAATCCC$  ${\tt ACCGTCCCACGGGGCAAGGGCCACTTGTGGGCCGGACCTCTAGCTGGATCTCCCCACCACGGA}$ GAAAAGTGGGCCGTGGGCCTCTCATTTCTTTCCCTTTGTATTGCTGGGCCGACTG  ${\tt GAGGGAATTTGGTCCATTGTACTGAGAGTCATATGCCCATTTCTTAGAAATTGTTCATTTGTC}$  ${\tt CCATATTACAAGTCCCTCTTTCTCAAAAGGTGTCATGTAAAAAATAATACGTTCCCTAATTAA}$  ${\tt AATTTTTATAGTAAAGAATATTTTTAAATTCTGGGTTTACATGATTATCGTATCATCTCTCCC}$ GGGAAGAAAAGAAAACGTACCTACGTAATGCTGTACAGCGTAATGCCAACTTTCGTACGAGA  ${\tt AAGCTGGGTCTAAAAAAAAACCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCC}$  ${\tt TTATTTAACTTGCTATTTCTAGCTCTAAAACCAACCATCAGCAGCACGTCGCAAGCTGAGCC}$  ${\tt TCAGCGCAGCAGCTTATAAGCAAGTGGTCGCGTCGTTGCGTGAGCGAGGTGGTACTAAACTGT}$  ${\tt TGTTTCCGCCCACGGGCTCTGCAATCCCACCGTCCCACGGGGCCACTTGTGGGCCGG}$  ${\tt ACCTCTAGCTGGATCTCCCCACCACGGAGAAAnGTGGGCCGTGGCCTGTGGgCCCCTCATTT}$  $\tt CTTTCCCTTTGTATTGCTGGGCCGACTGGAGGGAATTTGGTCCATTGTACTGAGAGTCATATG$ CCCATTTCTTAGAAATTGTTCATTTgTCCCATATTACAAGTCCCTCTTTCTCAAAAAGgtgTCA TGTAAAAAATATACGTTCCCTAATTAAAATTTTTATAGTAAagAATATTTTTAAAATTCTGG  $\verb|gtttaCatGATTATCGTntCATCTCCGGgAnnAAaannAAaaCGTACCTACGTAATGCTgn|\\$ nCgaattcGAGCGATCTAGTAa

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# Curriculum vitae Martin Hinrichs

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# **Scientific Publications**

# **Published Paper**

Fleck AT, Schulze S, Hinrichs M, Specht A, Waßmann F, Schreiber L, Schenk MK. 2015. Silicon promotes exodermal casparian band formation in Si-accumulating and Si-excluding species by forming phenol complexes. PLoS ONE 10, e0138555.

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Klotzbücher A, Klotzbücher T, Jahn R, Xuan LD, Cuong LQ, Van Chien H, Hinrichs M, Sann C, Vetterlein D. 2017. Effects of Si fertilization on Si in soil solution, Si uptake by rice, and resistance of rice to biotic stresses in Southern Vietnam. Paddy and Water Environment.

#### **Oral Presentations**

**Hinrichs M, Ngo NS, Schenk MK.** 2016. Silicon reduces Iron in Rice. Annual meeting of the German Society of Plant Nutrition, Hohenheim, Germany & Doctoral Researcher's Conference of GRK1798 "Signaling at the Plant-Soil Interface, Bad Salzdetfurth, Germany.

#### **Poster Presentations**

**Hinrichs M, Bremer M, Küster H, Schenk MK.** 2013. Functional analysis of candidate genes for P-starvation induced root hair growth, International Plant Nutrition Colloquium, Istanbul, Turkey

**Hinrichs M, Schenk MK. 2014.** Transcriptomic analysis of Si-induced Casparian band development in rice, 6<sup>th</sup> International Conference on Silicon in Agriculture, Stockholm, Schweden & Annual meeting of the German Society of Plant Nutrition, Halle, Germany.

**Hinrichs M, Schenk MK.** 2015. Transcriptomic data reveal new insights in Si effects on iron acquisition of rice. Rhizosphere 4" International Conference, Maastricht, Netherlands & Doctoral Researcher's Conference of GRK1798 "Signaling at the Plant-Soil Interface, Bad Salzdetfurth, Germany.

Hinrichs M, Fleck AT, Biedermann E, Ngo NS, Schreiber L, Schenk MK. 2017. An ABC Transporter is Involved in the Silicon-Induced Formation of Casparian Bands in the Exodermis of Rice. International Plant Nutrition Colloquium, Copenhagen, Denmark.